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# THE ESTIMATION OF THE STROKE VOLUME OF RATS FROM THE BODY WEIGHT AND HEART WEIGHT[1]

BY BERNARD GRAD

## Abstract

The object of this study was to arrive at useful empirical equations for the estimation of the stroke volume of rats from the body weight and heart weight. A logarithm of the $\frac{\text{oxygen consumption}}{\text{heart rate}}$ against body weight of rats weighing from 5 to 500 gm. gave a significant positive regression which permitted the estimation of the stroke volume from the body weight, assuming a certain constant value for the arterio-venous oxygen difference. A similar plot of the heart weight of rats against body weight gave a curve which was superimposable on the first one, allowing the estimation of the stroke volume from the heart weight.

## Introduction

The present report is based on a series of oxygen consumption and heart rate determinations taken on 176 Sherman rats from birth to old age (7). A plot of the ratio of the oxygen consumption over the heart rate against body weight on log paper gave a significant regression ($P < .001$, Fig. 1) and the following equation:

$$[I] \quad \frac{\text{O}_2 \text{ consumed (ml./min.)}}{\text{heart rate (beats/min.)}} = 2.47 \times 10^{-4} \text{ (body wt. (gm.)}^{0.75}$$

$$= \text{stroke volume (SV) (ml./beat)} \times \text{A-V O}_2 \text{ difference (A-V) (ml. O}_2\text{/ml. blood)}.$$

The following evidence indicates that the high regression of SV × A-V on body weight was due mainly to the change in SV with body weight: SV × A-V increased 91 times as the rats grew from 4.5 to 540 gm. (Fig. 1); assuming that the A-V for the rat was around 9.5 ml. $O_2$/100 ml. blood (see below), the A-V could not increase even threefold from the newborn to the adult in these animals, as that would involve the withdrawal from the blood of more oxygen than was originally present (100 ml. of adult rat blood contains 20.9 ml.

[1] *Manuscript received May 6, 1953.*

*Contribution from the Gerontologic Unit, Allan Memorial Institute, Department of Psychiatry, McGill University, Montreal, Canada.*

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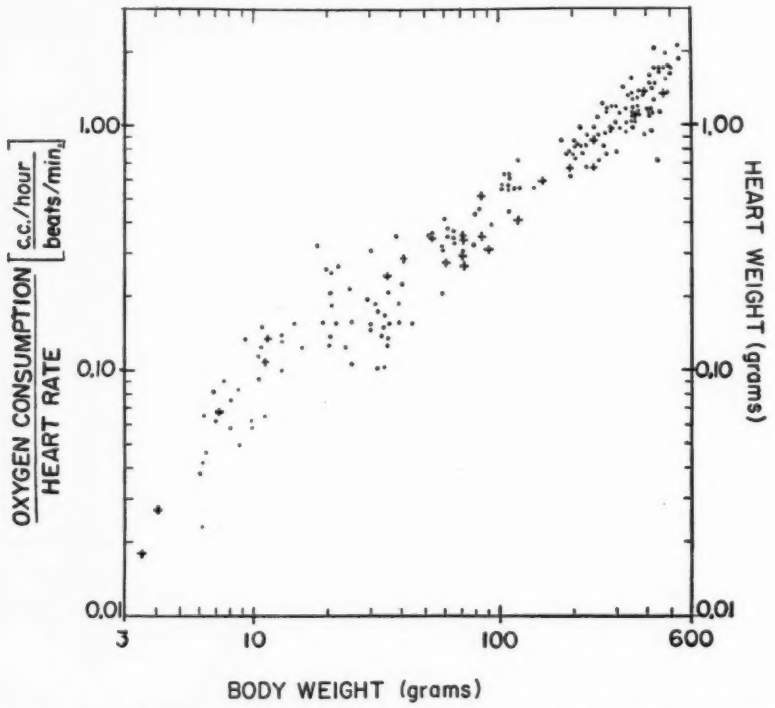


FIG. 1. The change in the oxygen consumption/heart rate and in the heart weight with body weight in rats.

● ● ● Oxygen consumption/heart rate.
+ + + Heart weight.

oxygen in combination with the 15.6 gm. of hemoglobin (5) plus about 0.24 ml. oxygen in solution (2) ). On the other hand, the capacity of the stroke volume to increase was much greater because it was a function of heart weight which actually increased 77-fold as the rats grew from 3.5 to 390 gm. (Fig. 1). There was no significant regression of A-V on body weight in mammals from the rat to the horse: ($P > 0.20$; see columns I and III of Table I for body weight and A-V data from rat to the horse). Yet in the same animals there was a significant regression of $\frac{O_2 \text{ consumption}}{\text{heart rate}}$, that is, SV × A-V, on body weight ($P < .001$; see colums I, II, and IV of Table I for body weight, oxygen consumption, and heart rate of these animals).

Therefore, the A-V can be considered as constant in rats of different weight. Hence, from equation I,

$$[II] \qquad SV = \frac{(2.47 \times 10^{-4} \text{ (body weight)}^{0.75})}{\text{A-V}}$$

TABLE I

BODY WEIGHT, OXYGEN CONSUMPTION, A-V OXYGEN DIFFERENCE, AND HEART RATE IN MAMMALS FROM THE RAT TO THE HORSE

| Animal | Body wt. (gm.) I | Total oxygen consumption (ml./min.) II | A-V oxygen difference (ml. $O_2$/ml. blood) III | Heart rate (beat/min.) IV |
|---|---|---|---|---|
| Rat | 190 (7) | 4.4 (7) | 0.095 (Present study) | 375 (7) |
| Rabbit | 3250 (9) | 27 (9) | 0.064 (10) | 225 (11) |
| Dog | 13,900 (13) | 92 (13) | 0.0495 (13) | 80 (13) |
| Goat | 23,600 (1) | 136 (1) | 0.0525 (1) | 75 (6) |
| Man | 65,000 (8) | 226 (8) | 0.062 (1) | 60 (8) |
| Horse | 500,000 (4) | 1447 (4) | 0.072 (12) | 38 (6) |

*Note: The number in parentheses gives the reference from which the adjoining figure was obtained.*

Blood, Smith, and D'Amour have shown that the cardiac output of rats weighing 180 to 200 gm. is 46.5 ml. per minute (3). The oxygen consumption of animals of this weight was around 265 ml. per hour (7). From this, the A-V may be calculated as being $\frac{265}{60 \times 46.5} = 0.095$ ml. oxygen per ml. of blood. Substituting this value for the A-V in equation [II], the stroke volume for the rat of any given weight can be estimated directly from the body weight as follows:

[III] stroke volume (ml./beats) $= 2.60 \times 10^{-3}$ (body weight (gm.) $)^{0.75}$ .

The stroke volume of rats of different weight calculated according to equation [III] is given in Table II.

TABLE II

THE ESTIMATION OF STROKE VOLUME OF RATS FROM THE BODY WEIGHT AND HEART WEIGHT

| Body wt. (gm.) | Heart wt. (gm.) | Stroke volume $2.60 \times 10^{-3}$ body wt.$^{0.75}$ (ml./beat) | Stroke volume $2.33 \times 10^{-1}$ heart wt. (ml./beat) |
|---|---|---|---|
| 7.3 | 0.068 | 0.012 | 0.016 |
| 190 | 0.669 | 0.13 | 0.16 |
| 465 | 1.322 | 0.26 | 0.31 |

The stroke volume of a 190 gm. rat can also be estimated from the $\frac{\text{cardiac output}}{\text{heart rate}}$ which equals $\frac{46.5}{375} = 0.12$ ml. per beat, 375 beats per minute being the heart rate of 180–200 gm. rats (7). The difference in the stroke volumes calculated according to equation [III] or from the cardiac output is 8%.

The stroke volume can also be calculated from the *heart weight* of rats. Utilizing the data of Fig. 1, the equation of heart weight versus body weight for a group of Sherman rats weighing from 3.5 to 465 gm. was

[IV] $\text{heart weight (gm.)} = 1.12 \times 10^{-2} (\text{body weight (gm.)})^{0.79}$ .

Inasmuch as the slope of the log of the SV × A-V versus log body weight is practically identical with that of the log heart weight versus log body weight, the following may be written from equations [I] and [IV]:

[V] $\text{stroke volume (ml./beat)} \times \text{A-V } O_2 \text{ difference (ml. } O_2\text{/ml. blood)} = 2.21 \times 10^{-2} \text{ heart weight}$ .

Substituting a value of 0.095 ml. $O_2$/ml. blood for the A-V $O_2$ difference, equation [V] becomes:

[VI] $\text{stroke volume (ml./beat)} = 2.33 \times 10^{-1} \text{ heart weight (gm.)}$ .

From equation [VI] estimations of the stroke volumes from rat hearts of different weight were obtained (Table II).

In summary, empirical equations * were derived for the approximation of stroke volume of rats from their body weight and heart weight.

* *Present-day statistical procedures do not provide a method for obtaining the error of estimate of the above equations in the form in which they stand, either because these equations were originally derived by transforming the variables to logarithms as in the case of equations [I] to [IV], or because they were derived from other equations which were so transformed as in equations [V] and [VI]. However, the standard error of the logarithm of equation [I] has been calculated and is as follows:*

$$\log \frac{O_2 \text{ consumed (ml./min.)}}{\text{heart rate (beats/min.)}} = -1.83 + 0.75 \text{ (log body weight)} \pm 0.11 .$$

The same standard error may be used for equations [II] and [III], assuming that the A-V remains constant. Using the logarithmic standard error of 0.11 for equation [III], two-thirds of a population of rats weighing around seven grams should have a stroke volume with the range 0.010 to 0.015 ml./beat, and the corresponding range for 190- and 465-gm. rats should be 0.10 to 0.17 ml./beat and 0.20 to 0.34 ml./beat respectively.

The logarithm of equation [IV] and its logarithmic standard has also been calculated:

$$\log \text{heart weight (gm.)} = -1.95 + 0.79 \text{ (log body weight)} \pm 0.09 .$$

## Acknowledgments

I am indebted to Dr. James W. Fisher of the Department of National Health and Welfare, Ottawa, and to Dr. C. W. Dunnett of the Food and Drug Division, Ottawa, for their comments on the statistics of this paper. I am indebted to Mr. Lionel Caplan for technical assistance.

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# TRAUMATIC RENAL FAILURE IN THE RAT[1]

By Kenneth S. Morton and Sydney M. Friedman

## Abstract

A systematic study of the variables connected with the production of renal damage following a crushing limb injury in the rat has been made. The state of hydration of the animal immediately prior to and following the crushing injury appears to be the most critical variable. Myoglobin, while it intensifies the renal dysfunction, is not necessary for its production. The area involved in the crush as well as the amount of renal reserve, are important factors. Various steroid hormones were ineffective in altering the course of the disease.

## Introduction

In 1941, Bywaters and Beall (1) noted that some of the casualties suffering crushing injuries during bombing raids developed signs of shock which went on to a picture of renal failure, uremia, and death. This condition they termed "crush syndrome" and since that time a considerable body of literature has accumulated from experimental, clinical, and historical sources. It is now generally appreciated that the syndrome is not new, having been described in connection with a wide variety of renal injuries (9). The ubiquitous nature of the condition is indicated by the multiplicity of names applied to it. "Traumatic anuria" is a clinical descriptive term, "lower nephron nephrosis" a pathological classification, and "renal anoxia syndrome" an etiological approach to classification.

Recently, Oliver's elegant technique (5) for studying the nephron in continuity has led him to a unifying concept which fits the observed clinical and pathological data. Oliver and his co-workers (6) have concluded that there are two essential lesions in the kidney of "acute tubular necrosis". These lesions are (i) nephrotoxic tubular necrosis; here the epithelium disintegrates between intact basement membranes, producing lesions restricted to the proximal convolution and evenly distributed in all nephrons of a damaged kidney, (ii) tubulorhexis; here there is localized destruction of the entire tubular wall such that tubular continuity is irreparably destroyed. This lesion may be found anywhere in a nephron and is patchily distributed throughout the kidney.

The considerable volume of experimental and clinical data suggests three possible pathogenetic mechanisms for acute tubular necrosis of either the "nephrotoxic" or "tubulorhectic" type; (*a*) simple obstruction of tubules by precipitated material, (*b*) toxins specifically injurious to the kidney, and (*c*) renal ischemia. The present report is concerned only with the first of these experimental approaches to the problem, and specifically deals with myoglobin as the precipitate.

[1] *Manuscript received August 19, 1953.*

*Contribution from the Department of Anatomy, The University of British Columbia, Vancouver, B.C.*

*This work was carried out under Grant No. 117 of the Defence Research Board of Canada.*

Experimental work by Corcoran and Page (3, 4) in rats, and Bywaters and Stead (2) in rabbits, has tended to substantiate the importance of myoglobin for the renal damage which follows muscle trauma. Both of these groups of investigators had used tourniquets applied to one or both hind limbs of the experimental animal, followed by the intravenous injection of myoglobin upon release of the ligature. In both instances, however, the multiplicity of simple variables seems to have received insufficient study. The major portion of the present report is concerned in the first instance with a stepwise reassessment of the various factors involved in the renal damage which is reported to follow muscle trauma (by tourniquet) and myoglobin administration.

In addition to this, certain studies in the past had indicated that testosterone was able to help protect the kidney from certain directly damaging procedures (8). It seemed of interest, therefore, to determine whether this or other similar steroids might ameliorate the renal damage caused by limb ligation. Testosterone, cortisone, desoxycorticosterone acetate, and 17-hydroxy corticosterone were tested. The results in all cases were negative.

## Methods

Albino rats of an inbred Wistar strain, weighing 200 to 350 gm. were used throughout these studies. Individual experiments were limited to 12 rats because of the obvious difficulties of timing procedures and studying urine output. In order to provide sufficient numbers of animals, certain experiments were repeated without change.

Myoglobin (Delta Chemical Co.) when used in early experiments, was dissolved in 0.9% saline and later in phosphate buffer at pH 7.35 and given intravenously into the femoral vein. The dose used ranged from 0.1 to 0.15 mgm./gm. body weight, based on studies of Bywaters and Stead (2) as well as those of Corcoran and Page (3). This dose required the injection of up to 1.6 cc. of total volume in a 250 gm. rat, but given slowly it appeared well tolerated. Injection was carried out under light ether anesthesia and in those experiments in which myoglobin was given to crushed animals the injection followed immediately after the release of the crushing ligature in order to simulate the condition expected in species with myoglobin-rich muscles.

Crushing injury was effected by applying limb ligatures according to the method of Corcoran and Page (3). It was found advisable to protect the ligature with a cover of adhesive tape. During the period of ligation the rats were kept sedated with 2.5 mgm. of nembutal per 100 gm. body weight, repeated if required. They were kept in metabolism cages with funnels to facilitate observation, handling, and urine collection. Ligatures were removed under nembutal supplemented, if necessary, with light ether; the limbs were massaged until the doughy consistency disappeared and the foot appeared bright pink; the rats were then returned to metabolism cages for further study.

Blood urea nitrogen (BUN) was determined by the method of Ormsby (7) in blood obtained by cardiac puncture with a 25 gauge needle. This procedure, in our hands, is rapid and permits proper sampling of blood without prolonged and undue handling of the animals. The procedure was applied uniformly to control as well as to test animals.

Kidney histology was studied in sections stained with hemalum and phloxine after fixation in Zenker's and, in later experiments, Herlant's fluid.

Because of the limitations of the rat in experiments such as reported here, the principal aim was to assess gross renal efficiency under each of the circumstances studied.

Because of the necessary limitation in the size of the groups studied, we feel that the calculation of standard error or standard deviation would give misleading impressions. Accordingly, range is presented instead and with the data more in the raw state, conclusions can be drawn only when the group data are broadly different.

## Experimental

*Experiment 1. The Effect of 48 hr. of Water Deprivation in Intact Male Rats*

This experiment was designed as a preliminary control step to determine the effect of water deprivation on the principal functions to be studied in these experiments. Of the 12 rats, four were untreated controls, four were deprived of water for 48 hr. and the remaining four, in addition to being deprived of water, were anesthetized with nembutal during the second 24 hr. dehydration period as control for the later use of nembutal. Table I presents the pertinent data.

It is evident that water deprivation even for this short period is reflected in a reduction in the appetite for solid food and a marked loss in weight. It is followed by a pronounced oliguria during the second day of dehydration but no change in BUN occurs. From our experience with the method, no significance can be attached to the difference between 40 and 50 mgm. % in BUN with the limited size of group. As expected, no histological change was noted in the kidneys. This pattern of response to water deprivation is not altered by interim nembutalization.

*Experiment 2. The Effect of 72 hr. of Water Deprivation in Uninephrectomized Male Rats*

Since, in later experiments, the response of animals with reduced renal reserve was to be studied, data were sought concerning the effects of simple water deprivation in previously uninephrectomized rats. Of the 12 rats in this experiment, four were untreated controls while eight, uninephrectomized four days before, were subjected to continuous water deprivation for 72 hr. Table II summarizes the pertinent data.

As in the preceding experiment, and more markedly so with this more prolonged period of dehydration, urine volume decreases progressively. This relative oliguria is accompanied by a tendency towards aciduria which is

TABLE I

THE EFFECT OF 48 HR. OF WATER DEPRIVATION IN INTACT MALE RATS

| Group | Total food intake, gm. | Total water intake, cc. | Body wt. change, gm. | Urine volume, cc. | | Urine pH | | BUN, mgm. % 48 hr. |
|---|---|---|---|---|---|---|---|---|
| | | | | 24 hr. | 48 hr. | 24 hr. | 48 hr. | |
| Control | 36.7 (30.2–42.3) | 45.9 (31.5–56.0) | + 7.5 (0– + 14) | 9.3 (7.9–12.3) | 6.8 (3.5–11.4) | 6.6 (6.5–7.0) | 6.5 (6.5–6.5) | 40 (40–40) |
| Water deprivation | 1.5 (0.0–6.0) | 0 | − 33 (− 30– − 36) | 8.6 (4.8–13.2) | 2.6 (1.6–3.4) | 6.4 (6.0–6.5) | 6.2 (6.0–6.5) | 50 (50–50) |
| Water deprivation plus nembutal | 16.0 (15.0–16.5) | 0 | − 26 (− 22– − 28) | 7.2 (6.4–8.6) | 2.6 (2.2–3.0) | 6.9 (6.5–7.0) | 6.5 (6.5–6.5) | 52 (50–60) |

TABLE II

THE EFFECT OF 72 HR. OF WATER DEPRIVATION IN UNINEPHRECTOMIZED MALE RATS

| Group | Urine vol., cc. | | | Urine pH | | | BUN, mgm. % 72 hr. |
|---|---|---|---|---|---|---|---|
| | 24 hr. | 48 hr. | 72 hr. | 24 hr. | 48 hr. | 72 hr. | |
| Control | 10.4 (10.0–10.8) | 10.2 (6.0–14.2) | 9.7 (6.8–12.1) | 6.7 (6.5–7.0) | 6.9 (6.5–7.5) | 7.0 (7.0–7.0) | 70 (60–80) |
| Water deprivation | 5.1 (3.0–6.4) | 1.3 (0.4–2.0) | 0.9 (0.2–1.6) | 6.9 (6.5–7.0) | 6.0 (5.5–6.5) | 6.0 (6.0–6.0) | 97 (80–120) |

significant in the last period. The attempt of the kidney to conserve water is also reflected in the slight but significant rise in the blood urea nitrogen in the last period. No histological change occurred in the kidney.

In summary, water deprivation produces no structural change in the kidney and is well tolerated for as long as 72 hr. even in rats with reduced renal reserve. It induces (*a*) a diminished appetite for solid food, (*b*) a progressive decline in urine output, (*c*) a loss in body weight, (*d*) a tendency towards aciduria and, if sufficiently prolonged in rats with reduced renal reserve, it may also lead to (*e*) a minor degree of azotemia.

*Experiment 3. The Effect of Intravenous Myoglobin in Intact Male Rats*

This experiment was designed to study the effect of intravenously injected myoglobin (as described in methods) on urine output, BUN, and kidney histology. The experiment was carried out in three sections using 12 rats in each. In the first, four rats served as untreated controls, four received intravenous saline (0.9%) in an amount and by a technique identical with those of the third group of four animals, which received myoglobin. The myoglobin administered (0.1 mgm./gm. body weight) was dissolved and suspended in 1 cc. of saline. In the second section the procedures and groups of the first were duplicated exactly. In the third section the dose of myoglobin was increased to 0.15 mgm./gm. body weight. Table III summarizes the pertinent data.

The results of all three sections of this experiment were entirely consistent in showing that intravenous injection of myoglobin in the dosages used here was without effect on either urine output or BUN values. Further, no histological change was encountered in the kidney of any of the animals.

*Experiment 4. The Effect of Intravenous Myoglobin in Male Rats Deprived of Water*

Four of the 12 rats of this experiment were deprived of water continuously for 72 hr. as controls. The remaining eight rats were similarly dehydrated but, in addition, after 24 hr. of water deprivation, received myoglobin (0.15 mgm./gm. body weight in saline) intravenously. The pertinent data are presented in Table IV.

As expected, prolonged dehydration caused a marked oliguria and a tendency towards aciduria. Even in the presence of these renal compensations intravenously injected myoglobin failed to aggravate the findings, nor did it cause any elevation of BUN or alteration in renal histology.

*Experiment 5. The Effect of "Tourniquet Crush" to One Hind Limb in the Male Rat*

Four animals served as untreated controls in this experiment, while the remaining eight were subjected to tight ligation of one hind limb (see methods) applied for five hours. Since the ligated animals were kept sedated with nembutal, similar sedation was also given the controls. It was assumed that

## TABLE III

THE EEFECT OF INTRAVENOUS MYOGLOBIN IN INTACT MALE RATS

| Group | Total food intake, gm. | Total water intake,cc. | Body wt. change, gm. | Urine volume, cc. | | Urine pH | | BUN, mgm. % | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 48 hr. | 72 hr. | 48 hr. | 72 hr. | 24 hr. | 48 hr. | 72 hr. |
| *Section 1* | | | | | | | | | | |
| Control | 43.4 (41.5–45.5) | 55.6 (49.0–64.0) | + 11 (4–18) | 8.8 (7.4–10.5) | 7.8 (4.8–9.4) | 6.4 (6.0–6.5) | 6.9 (6.5–7.0) | — | — | 55 (40-70) |
| Saline control | 21.0 (19.0–23.0) | 29.5 (26.5–32.5) | − 20 (0– −40) | 10.2 (2.2–12.6) | 8.7 (2.4–10.3) | 6.8 (6.0–7.5) | 6.4 (6.0–7.0) | — | — | 60 (50–70) |
| Myoglobin | 24.1 (20.0–28.0) | 27.0 (18.5–36.0) | − 13 (− 8– −16) | 5.8 (4.2–6.2) | 3.9 (2.8–5.0) | 6.9 (6.5–7.0) | 6.8 (6.5–7.0) | — | — | 60 (50–70) |
| *Section 2* | | | | | | | | | | |
| Control | 40.7 (38.0–42.0) | 55.6 (50.0–58.0) | — | 7.9 (7.0–10.1) | 5.8 (3.8–9.0) | 6.8 (6.5–7.0) | 7.1 (6.5–7.5) | 62 (60–70) | 65 (60–70) | 59 (50–60) |
| Saline control | 44.5 (39.0–47.5) | 62.3 (57.0–69.5) | — | 7.4 (4.4–9.6) | 5.0 (3.0–6.1) | 6.4 (6.0–7.0) | 7.3 (6.5–7.5) | 62 (50–70) | 62 (50–80) | 65 (50–80) |
| Myoglobin | 39.5 (26.5–44.5) | 66.1 (54.0–87.0) | — | 8.9 (6.6–11.5) | 11.1 (6.6–15.4) | 6.6 (6.5–7.0) | 7.4 (7.0–7.5) | 65 (60–70) | 65 (60–70) | 52 (50–60) |
| *Section 3* | | | | | | | | | | |
| Control | 40.0 (37.0–41.0) | 54.0 (47.0–59.0) | + 13 (+ 8– +18) | 5.3 (3.2–9.2) | 3.9 (3.5–4.6) | 6.4 (6.0–6.5) | 6.9 (6.5–7.5) | 62 (60–70) | 67 (60–70) | 55 (50–60) |
| Saline control | 39.0 (34.0–40.0) | 64.0 (53.0–73.0) | + 9 (+ 8– +10) | 9.2 (4.2–14.2) | 6.9 (4.0–9.0) | 6.6 (6.5–7.0) | 7.3 (6.5–7.5) | 68 (60–70) | 70 (60–80) | 60 (50–70) |
| Myoglobin | 33.0 (25.0–40.0) | 55.0 (40.0–63.0) | + 6 (− 1– +14) | 6.4 (4.6–9.6) | 6.1 (3.8–8.6) | 6.8 (6.5–7.0) | 7.3 (6.5–7.5) | 65 (60–70) | 65 (60–70) | 52 (50–60) |

the ligated rats would probably not drink much, and accordingly an attempt, unsuccessful in this first trial, was made to match the intake of the controls to that of the treated animals.

In order to provide information concerning kidney histology after crush, two animals of the test group were sacrificed at 24 hr., two at 48 hr. and the remainder, together with the control rats, at 72 hr. Table V summarizes the data.

The crushing injury to only one limb produced striking and somewhat unexpected results. To begin with, the water intake of the controls was arbitrarily reduced to match an expected oliguria in the crushed rats. Since this did not occur, the control rats were in effect relatively dehydrated in this experiment. Surprisingly, following crush, the rats showed a marked water hunger, drinking in 24 hr. about twice as much as normal rats. In the same period these crushed rats put out six times as much urine as the controls. This polyuria continued with diminishing intensity over the next 48 hr. and is clearly evident even in those cases where the water intake of the controls is well matched to the treated. The polyuria was accompanied by a definite progression towards an alkaline urine. This polyuria, which will be discussed later, was a recurrent phenomenon in the experiments which follow and is considered to be an early manifestation of renal damage. A minimal but not significant tendency towards azotemia may possibly have occurred in the first 24 hr. period following crush.

Postmortem findings were essentially negative. There was no gross evidence of kidney damage. The crushed limb was swollen, cold, pale to bluish in color, and edematous. Histological findings in the kidney were not remarkable.

*Experiment 6. The Effect of "Tourniquet Crush" to One Hind Limb in Male Rats Deprived of Water*

This experiment was designed to study the effect of crushing one limb, a procedure known to cause a minimal renal functional change, in animals whose renal function was already under the strain of water deprivation. The experiment was carried out in two sections of 12 rats each, in order to increase the number of observations.

All the rats were continuously deprived of water for 72 hr. In each section four rats served as controls while in the remaining eight a tourniquet was applied to one hind limb as in the preceding experiment. The tourniquet was applied 24 hr. after the start of water deprivation, and the rats were followed for three days thereafter. Accordingly, in the table, the last recorded observation occurred after 24 hr. of free access to water. Table VI presents the data.

Both sections yielded substantially the same results, the only difference being that the dehydrated controls of the first section maintained body weight, while in the second section marked weight loss occurred in this group. In both sections the crushed animals lost more weight than their controls. Again

## TABLE IV

THE EFFECT OF INTRAVENOUS MYOGLOBIN IN MALE RATS DEPRIVED OF WATER

| Group | Total food intake, gm. | Body wt. change, gm. | Urine volume, cc. | | Urine pH | | BUN, mgm. % | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | 48 hr. | 72 hr. | 48 hr. | 72 hr. | 24 hr. | 48 hr. | 72 hr. |
| Control dehydration only | 19.0 | − 3 | 0.12 | 2.3 | 6.0 | 6.0 | 87 | 75 | 65 |
| | (11.0–24.0) | (− 14– + 6) | (0.1–0.2) | (1.0–2.6) | (6.0–6.0) | (6.0–6.0) | (70–120) | (70–90) | (60–70) |
| Myoglobin plus dehydration | 22.0 | + 2 | 0.33 | 2.7 | 6.1 | 5.9 | 90 | 82 | 69 |
| | (16.0–29.0) | (− 4– + 10) | (0.1–1.0) | (0.8–3.8) | (6.0–6.5) | (5.5–6.5) | (50–140) | (60–100) | (60–70) |

## TABLE V

THE EFFECT OF "TOURNIQUET CRUSH" TO ONE HIND LIMB IN THE MALE RAT

| Group | Total food intake, gm. | Total water intake, cc. | | | Body wt. change, gm. | Urine volume, cc. | | | Urine pH | | | BUN, mgm. % | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 24 hr. | 48 hr. | 72 hr. | | 24 hr. | 48 hr. | 72 hr. | 24 hr. | 48 hr. | 72 hr. | 24 hr. | 24 hr. | 72 hr. |
| Control | 37 | 11.0 | 19.0 | 18.0 | − 24 | 3.4 | 3.4 | 3.6 | 7.0 | 6.6 | 6.6 | 82 | 90 | 67 |
| | (35–43) | (7.0–15.0) | (12.0–24.0) | (3.0–31.0) | (− 14– − 30) | (2.1–4.7) | (1.4–4.7) | (2.4–4.8) | (6.5–7.5) | (6.5–7.0) | (6.0–7.0) | (70–90) | (70–110) | (60–80) |
| Crushed | 8 | 32.0 | 16.0 | 33.0 | − 36 | 23.0 | 15.4 | 9.5 | 6.4 | 7.4 | 7.3 | 96 | 83 | 67 |
| | (0–29) | (20.0–48.0) | (6.0–32.0) | (20.0–42.0) | (− 22– − 48) | (12.2–31.2) | (7.5–26.5) | (6.4–17.2) | (6.0–6.5) | (7.0–7.5) | (7.0–7.5) | (80–110) | (70–100) | (50–80) |

the ligated animals showed, as they had in experiment 5, a relative polyuria. Urine output was, of course, low in all groups as a consequence of dehydration but consistently higher in crushed rats. This relative polyuria became a real polyuria in the last 24 hr. when free access to water was allowed.

During the period of dehydration, as noted in earlier experiments, the urine tends to become more acid. On the other hand, the crushing injury tends to result in a more alkaline urine (as seen before) and was observed here only when free access to water was allowed.

Definitely uremic blood levels were seen in the treated animals of both sections of this experiment indicating how greatly water deprivation may affect the results of a crushing injury. In accord with the uremia of the 16 test animals, two died before the kidney could be properly fixed and eight showed minimal to moderate renal damage. In these the cells of the proximal tubule appeared intact whereas distal tubules and especially the thick portion of Henle's loop showed early degenerative changes. These consisted of granular vacuolated cytoplasm; swollen, pale, and vacuolated nuclei; and occasionally, a pyknotic nucleus. Desquamated cells were frequent.

Thus, crush injury in dehydrated animals caused a relative renal failure. In the stages observed in this experiment, water output was increased rather than decreased. It will appear later that the oliguria which had been expected is a response characteristic only of a greater degree of damage.

*Experiment 7. The Effect of "Tourniquet Crush" to One Hind Limb in Male Rats Receiving Intravenous Myoglobin*

Previous experiments had shown that a crushing injury in the form of a tourniquet applied to one hind limb caused a mild renal dysfunction and that this could be severely aggravated by dehydration. Accordingly, it seemed important to determine whether myoglobin (which had figured prominently in the work of other investigators), like dehydration, would aggravate the renal effect of the crush injury.

Of the 12 rats in this experiment, four served as untreated controls, while the remaining eight were subjected to the usual tourniquet crush. Four of these ligated animals were given intravenous myoglobin (0.15 mgm./gm. body weight) immediately after removal of the ligature, while the remaining four ligated animals received the buffer solution used as solvent for the myoglobin. Table VII lists the pertinent data. An attempt was made in this experiment to match the food and water intake of the controls to that of the test animals.

Again, as in previous experiments, the diuretic response to the limb crush is evident and in no way modified by the injection either of myoglobin or of the buffer solution alone. Similarly, the minimal elevation in BUN following limb ligation, which was observed previously, occurred here without being aggravated by the additional injections.

This experiment, like the previous ones in which myoglobin had been injected in intact and again in dehydrated rats, indicates no specific renal damaging effect with myoglobin, at least in the dosage used here.

## TABLE VI

THE EFFECT OF "TOURNIQUET CRUSH" TO ONE HIND LIMB IN MALE RATS DEPRIVED OF WATER

| Group | Total food last 24 hr., gm. | Total water last 24 hr., cc. | Body wt. change, gm. | Urine volume, cc. | | | Urine pH | | | BUN, mgm. % | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 24 hr. | 48 hr. | 72 hr. | 24 hr. | 48 hr. | 72 hr. | 24 hr. | 48 hr. | 72 hr. |
| *Section 1* | | | | | | | | | | | | |
| Dehydrated control | 10.0 (7–12) | 30 (22–33) | 0 (+ 2– − 2) | 1.2 (0.4–2.5) | 0.9 (0.6–1.2) | 4.5 (1.6–5.6) | 6.2 (6.0–6.5) | 6.1 (6.0–6.5) | 6.0 (6.0–6.0) | 90 (80–100) | 67 (50–90) | 55 (50–60) |
| Dehydrated plus crush | 0.5 (0–4) | 21 (0–31) | − 24 (− 12– − 34) | 2.2 (1.2–3.4) | 6.9 (4.3–8.7) | 12.9 (6.9–15.4) | 5.9 (5.5–6.0) | 6.1 (6.0–6.5) | 7.0 (6.5–7.5) | 200 (140–280) | 176 (130–230) | 127 (110–160) |
| *Section 2* | | | | | | | | | | | | |
| Dehydrated control | 6.0 (0–9) | 22 (11–29) | − 39 (− 30– − 50) | 1.4 (0.6–1.8) | 0.3 (0.0–0.8) | 2.2 (0.6–4.0) | 6.0 (6.0–6.0) | 6.0 (6.0–6.0) | 6.0 (5.5–6.5) | 85 (80–100) | 107 (100–120) | 77 (70–90) |
| Dehydrated plus crush | 0.3 (0–1) | 21 (0–35) | − 47 (− 32– − 62) | 2.2 (0.0–2.8) | 3.1 (1.0–4.7) | 13.4 (8.0–24.5) | 6.0 (6.0–6.0) | 5.8 (5.5–6.0) | 7.0 (6.5–7.5) | 245 (130–440) | 190 (110–280) | 152 (90–250) |

## TABLE VII

THE EFFECT OF "TOURNIQUET CRUSH" TO ONE HIND LIMB IN MALE RATS RECEIVING INTRAVENOUS MYOGLOBIN

| Group | Total food intake, gm. | Total water intake, cc. | Body wt. change, gm. | Urine volume, cc. | | | Urine pH | | | BUN, mgm. % | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 24 hr. | 48 hr. | 72 hr. | 24 hr. | 48 hr. | 72 hr. | 24 hr. | 48 hr. | 72 hr. |
| Control | 27 (25–28) | 47 (39–57) | − 7 (+ 6– − 16) | 4.8 (3.8–6.5) | 4.9 (2.3–6.8) | 6.1 (3.6–8.6) | 6.5 (6.0–7.0) | 6.0 (6.0–6.0) | 6.3 (6.0–7.0) | 100 (90–110) | 73 (70–80) | 78 (60–90) |
| Ligation plus buffer | 12 (4–19) | 49 (18–75) | − 15 (− 2– − 24) | 14.2 (6.0–23.4) | 10.9 (8.0–16.5) | 7.9 (4.2–12.4) | 6.2 (6.0–6.5) | 7.1 (7.0–7.5) | 6.3 (6.0–7.0) | 112 (110–120) | 85 (80–90) | 86 (80–100) |
| Ligation plus myoglobin | 21 (19–23) | 58 (55–62) | − 7 (− 2– − 14) | 12.0 (10.2–14.4) | 8.7 (6.4–12.0) | 5.9 (4.2–8.7) | 6.0 (6.0–6.0) | 7.0 (6.5–7.5) | 6.8 (6.0–7.5) | 120 (120–120) | 85 (80–90) | 80 (80–80) |

*Experiment 8. The Effect of Intravenous Myoglobin on the Kidney in the Presence of "Tourniquet Crush" in Dehydrated Male Rats*

Previous experiments had demonstrated that definite renal injury could be produced by "tourniquet crush" applied to one limb in dehydrated animals. It remained to be determined whether myoglobin, which hitherto had produced no specific renal damage, would aggravate the injury produced by the other two factors.

Of the 12 animals in this experiment, four served as controls subjected to water deprivation only, four were subjected to tourniquet crush (as before) in the presence of water deprivation, and four received, in addition, intravenous myoglobin as in previous experiments. The pertinent data are presented in Table VIII. As before, since 72 hr. of water deprivation was started 24 hr. before the ligating procedure, which was considered as zero time, the rats were allowed free access to water during the last 24 hr. of the experiment.

In this experiment dehydration alone resulted in a diminution in urine output, and a rising BUN followed by rapid restoration on the last day of the experiment when free access to water was allowed. The addition of the ligation injury resulted in the usual diuretic response to the trauma and at the same time a marked rise in BUN. The addition of myoglobin to the trauma in these dehydrated rats caused a definite increase in renal damage as indicated by both a more marked diuretic response and a still greater degree of elevation in BUN.

Histological evidence of renal damage as reported in experiment 6 was again observed here in both ligated groups. The histological damage was not markedly greater in the group receiving myoglobin although occasional casts were seen.

*Experiment 9. The Effect of Bilateral Limb Ligation in Dehydrated Male Rats*

In this experiment all animals were deprived of water for 24 hr. prior to limb ligation, and for 48 hr. thereafter. Free access to water was allowed during the last 24 hr. of the experiment. Four of the 12 rats of this experiment were subjected to no further interference beyond the simple dehydration; four animals were subjected to ligation of one hind limb as previously described; four were subjected to bilateral hind limb ligation.

The pertinent data are presented in Table IX.

As anticipated, bilateral ligation considerably increased the severity of the trauma. While only one of the four rats subjected to single ligation died during the experiment, three of the four rats in the doubly ligated group died within 36 hr. The important point to decide, however, is whether these latter animals died because of renal failure or because of generalized shock. A complete answer cannot be given. It seems that increasing the area of crush did indeed increase the severity of renal insult to the point where three of the four rats died in anuria, while the fourth survived with a persistent elevation of blood urea nitrogen. Complete renal failure does not ordinarily

## TABLE VIII

THE EFFECT OF INTRAVENOUS MYOGLOBIN ON THE KIDNEY IN THE PRESENCE OF "TOURNIQUET CRUSH" IN DEHYDRATED MALE RATS

| Group | Total food last 24 hr., gm. | Total water last 24 hr., cc. | Urine volume, cc. | | | Urine pH | | | BUN, mgm. % | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 24 hr. | 48 hr. | 72 hr. | 24 hr. | 48 hr. | 72 hr. | 24 hr. | 48 hr. | 72 hr. |
| Dehydration | 10 (10–10) | 27 (21–39) | 1.9 (1.1–2.4) | 1.1 (0.6–1.6) | 3.8 (1.2–10.1) | 6.0 (6.0–6.0) | 6.0 (6.0–6.0) | 5.9 (5.5–6.0) | 80 (60–90) | 110 (90–140) | 65 (60–70) |
| Dehydration plus ligation | 8 (5–10) | 39 (33–44) | 2.3 (2.0–3.2) | 4.9 (3.4–7.2) | 8.6 (6.4–10.4) | 6.0 (6.0–6.0) | 6.4 (6.0–7.0) | 7.0 (6.0–7.5) | 160 (130–170) | 130 (110–150) | 85 (80–90) |
| Dehydration plus ligation plus myoglobin | 2.5 (0–7) | 39 (25–59) | 3.2 (2.3–4.9) | 7.5 (5.0–9.8) | 13.5 (9.2–15.8) | 6.0 (6.0–6.0) | 6.3 (6.0–7.0) | 7.2 (7.0–7.5) | 220 (160–290) | 185 (150–220) | 125 (100–160) |

## TABLE IX

THE EFFECT OF BILATERAL LIMB LIGATION IN DEHYDRATED MALE RATS

| Group | Total food last 24 hr., gm. | Total water last 24 hr., cc. | Wt. change, gm. | Urine volume, cc. | | | Urine pH | | | BUN, mgm. % | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 24 hr. | 48 hr. | 72 hr. | 24 hr. | 48 hr. | 72 hr. | 24 hr. | 48 hr. | 72 hr. |
| Dehydration | 10 (10–10) | 30 (24–36) | − 31 (−28– −38) | 1.4 (0.5–2.2) | 0.9 (0.3–1.8) | 2.8 (1.9–3.4) | 6.2 (6.0–7.0) | 6.0 (6.0–6.0) | 6.5 (6.0–7.0) | 107 (90–130) | 125 (100–180) | 77 (70–90) |
| Dehydration plus single ligation | 4 (0–10) | 30 (15–39) 1 of 4 dead | − 36 (−30– −46) | 2.8 (1.5–4.1) | 5.2 (0.0–8.1) | 9.5 (9.0–11.3) | 6.0 (6.0–6.0) | 6.8 (6.5–7.0) | 7.2 (7.0–7.5) | 237 (140–410) | 136 (120–150) | 120 (90–160) |
| Dehydration plus double ligation | 0 | 3 * | − 29 (−28– −32) | 1.9 (0.7–2.8) | 5.1 * | 13.3 * | 6.0 (6.0–6.0) | 6.5 * | 7.5 * | 237 (190–290) | 220 * | 220 * |

* *Figure based on one survivor only.*

kill a rat in 36 hr. however, so that one is forced to conclude that while the trauma caused specific renal damage, death resulted from the more generalized shocking effects of the trauma.

*Experiment 10. The Effect of a Reduction in Renal Reserve on the Response to Limb Ligation in Dehydrated Male Rats*

In view of the findings in the ninth experiment, it seemed pertinent to study the effect of unilateral limb ligation in animals with limited renal reserve.

This experiment was carried out in two sections. The 12 rats of the first section were unilaterally nephrectomized one week prior to the ligating procedure, while those of the second section were similarly treated three days before ligation. All animals in both experiments were deprived of water for 24 hr. prior to ligation and for 48 hr. thereafter. The minor effect of simple dehydration in uninephrectomized rats has already been noted. Section 1 was followed for 72 hr. after ligation while section 2 was designed for study of the effects during the first 36 hr. after operation. Table X presents the data.

The results of the two experimental sections were similar and will be considered together. There is little doubt that a preliminary reduction in renal reserve causes a marked increase in the renal response to limb crush. This can even be noted in a graded way, for while in the first section only one rat died and then only during the third day, in the second section, with rats uninephrectomized only three days before crush, two rats died within 36 hr.

That a primary effect of limb crush in a dehydrated animal is exerted on the kidney seems beyond doubt. Uremia of a marked degree occurred uniformly in the test groups. BUN (blood urea nitrogen) levels over 400 mgm. % were common, while one animal in section one reached 530 mgm. % and one in section two reached 600 mgm. %.

The relative polyuria as a species peculiarity in the rat appears again, as it has in all previous experiments in company with the elevated BUN, a finding which must indicate excessively sieve-like tubules. It should be noted, however, that those animals which died, were oliguric and then anuric in the terminal 10 to 20 hr.

It seems then that "crush syndrome" in the rat is characterized by a raised BUN with a relative polyuria followed, only when excessively severe, by anuria.

Marked tubular damage was uniformly seen in the kidneys of the test animals.

At this time preliminary studies were undertaken to determine whether certain steroids with known renal effects would alter the course of "crush syndrome" in the rat.

*Experiment 11. The Effect of Testosterone Propionate on the Renal Damage Caused by Limb Ligation*

All animals used in this study were subjected to unilateral nephrectomy four days prior to ligation. All were deprived of water for 24 hr. prior to ligation and 48 hr. thereafter. One limb was ligated for five hr. as before.

## TABLE X

THE EFFECT OF A REDUCTION IN RENAL RESERVE ON THE RESPONSE TO LIMB LIGATION IN DEHYDRATED MALE RATS

| Group | Total food last 24 hr., gm. | Total water last 24 hr., cc. | Wt. change, gm. | Urine volume, cc. | | | Urine pH | | | BUN, mgm. % | | | Deaths |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 24 hr. | 48 hr. | 72 hr. | 24 hr. | 48 hr. | 72 hr. | 24 hr. | 48 hr. | 72 hr. | |
| *Section 1* | | | | | | | | | | | | | |
| Dehydration | 5 (5–5) | 29 (27–31) | − 20 (−14–−26) | 1.6 (1.2–2.2) | 1.0 (0.6–1.7) | 8.0 (6.0–9.8) | 6.0 (6.0–6.0) | 5.9 (5.5–6.0) | 7.1 (7.0–7.5) | 160 (110–240) | 110 (90–170) | 95 (80–110) | — |
| Dehydration plus ligation | 1 (0–5) | 27 (3–46) | − 28 (−14–−44) | 1.6 (1.0–2.2) | 3.2 (0.6–7.4) | 16.3 (5.1–29.3) | 6.3 (6.0–7.0) | 6.8 (6.0–7.5) | 7.5 (7.5–7.5) | 320 (200–530) | 200 (130–240) | 190 (90–310) | 1 |
| *Section 2* | | | | | | | | | | | | | |
| Dehydration | — | — | − 38 (−34–−42) | 1.6 (1.1–1.9) | — | — | — | — | — | 105 (100–110) | 36 hr. 85 (80–90) | — | — |
| Dehydration plus ligation | — | — | − 31 (−18–−40) | 2.4 (1.7–3.1) | — | — | — | — | — | 285 (190–600) | 260 (200–310) | — | 2 |

This procedure of experiment 10 was selected in order to produce severe and possibly lethal renal damage so that it could be determined whether any sterioid could, under these circumstances, effect improvement.

This experiment was carried out in three sections. In each section of 12 rats, six served as controls subjected to the damaging procedure only, while six received the steroid for some days prior to it. Since testosterone in oil was used it was necessary to inject the steroid prior to operation in order to allow time for it to become effective.

The test animals in section 1 were adult male rats given 5 mgm. of testosterone propionate in 0.5 cc. sesame oil, three days and again one day prior to limb ligation.

The test animals of section 2 were adult female rats of the same strain and average weight as before. Testosterone was given as in section 1.

The test animals of section 3 were adult female rats of the same strain and average weight as before. Testosterone propionate, 5 mgm. in oil and 5 mgm. suspended in saline was administered subcutaneously at the time of ligation.

Table XI presents the pertinent data.

The results of the three sections of this experiment were uniform. The procedure as tested in experiment 10 turned out, on repetition here, to be very severe. In this experiment it killed most of the rats on the first, second, or third day. Since oliguria and even anuria was the rule prior to death, and a high BUN was noted whenever a satisfactory blood sample was obtained, it is reasonable to term the condition an experimental "acute traumatic anuria".

In no case was testosterone at all effective in ameliorating the course of the disease. That the steroid was active is indicated by the fact that in each section independently, the testosterone treated rats lost less weight than their controls, a reflection of the protein-sparing effect of the hormone.

*Experiment 12. The Effect of Cortisone Acetate on the Renal Damage Caused by Limb Ligation*

All animals used in this study were handled in the same way as those of the preceding experiment in so far as preliminary uninephrectomy and subsequent dehydration with limb ligation were concerned. The experiment was carried out in two sections. In each section, six rats served as controls subjected to the damaging procedure only, while six received the steroid as suspended microcrystals (Merck). Two milligrams of cortisone acetate was injected subcutaneously 24 hr. prior to ligation and repeated daily until termination.

Section one consisted of adult male, and section two, adult female, rats. Table XII presents the data, this time chiefly concerned with mortality rate.

As in the preceding experiment with testosterone, the test condition was very severe leading to death in anuria with an elevated BUN in all test animals. Cortisone acetate under these circumstances was completely incapable of altering the course of the disease.

## TABLE XI

THE EFFECT OF TESTOSTERONE PROPIONATE ON THE RENAL DAMAGE CAUSED BY LIMB LIGATION

| Group | Wt. change, gm. | Urine volume, cc. | | | Urine pH | | BUN, mgm. % at death | Deaths |
|---|---|---|---|---|---|---|---|---|
| | | 24 hr. | 48 hr. | 72 hr. | 24 hr. | 72 hr. | | |
| *Section 1* | | | | | | | | |
| Uninephrectomy, ligation, dehydration | − 22<br>(− 20− − 24) | 0.3<br>(0.1–0.5) | 3.4<br>* | 30.0<br>* | — | — | 310<br>* | 5 on 1st day |
| Uninephrectomy, ligation, dehydration, testosterone | − 16<br>(− 14− − 18) | 0.1<br>(0.0–0.4) | — | — | — | — | — | 6 on 1st day |
| *Section 2* | | | | | | | | |
| Uninephrectomy, ligation, dehydration | − 24<br>(− 20− − 28) | 0.8<br>(0.2–1.2) | 0.0<br>(2 animals) | — | 6.8<br>(6.0–7.0) | — | — | 3 on 1st day<br>2 on 2nd day<br>1 accidental |
| Uninephrectomy, ligation, dehydration, testosterone | − 20<br>(− 18− − 26) | 0.5<br>(0.2–0.9) | 0.0<br>(2 animals) | — | 6.1<br>(6.0–7.0) | — | 300<br>(2 animals) | 4 on 1st day<br>2 on 2nd day |
| *Section 3* | | | | | | | | |
| Uninephrectomy, ligation, dehydration | − 23<br>(− 18− − 28) | 1.0<br>(0.7–1.3) | 0.3<br>* | 24.5<br>* | 6.0<br>(6.0–6.0) | 7.0<br>* | 240<br>(2 animals) | 1 on 1st day<br>2 on 2nd day<br>2 on 3rd day |
| Uninephrectomy, ligation, dehydration, testosterone | −18<br>(− 6− − 24) | 0.8<br>(0.3–1.8) | 0.6<br>* | 26.0<br>* | 6.0<br>(6.0–6.0) | 7.5<br>* | 260<br>(4 animals) | 3 on 1st day<br>1 on 3rd day<br>1 accidental |

* *Single determination.*

TABLE XII

THE EFFECT OF CORTISONE ACETATE ON THE RENAL DAMAGE CAUSED BY LIMB LIGATION

| Group | Wt. change, gm. | Urine volume, cc. | | BUN, mgm. % at death | Deaths |
|---|---|---|---|---|---|
| | | 24 hr. | 48 hr. | | |
| *Section 1* | | | | | |
| Uninephrectomy, ligation, dehydration | − 17 (− 14– − 22) | 0.3 (0.1–0.5) | — | — | 6 on 1st day |
| Uninephrectomy, ligation, dehydration, cortisone | − 19 (− 16– − 22) | 0.6 (0.3–0.9) | — | — | 6 on 1st day |
| *Section 2* | | | | | |
| Uninephrectomy, ligation, dehydration | − 26 (− 20– − 30) | 0.8 (0.6–1.2) | 0.0 | 360 (270–400) 3 animals | 1 on 1st day 5 on 2nd day |
| Uninephrectomy, ligation, dehydration, cortisone | − 23 (− 18– − 34) | 0.8 (0.4–1.4) | 0.0 | 320 (190–500) 5 animals | 1 on 1st day 5 on 2nd day |

TABLE XIII

THE EFFECT OF CORTISONE ACETATE AND TESTOSTERONE PROPIONATE IN COMBINATION ON THE RENAL DAMAGE CAUSED BY LIMB LIGATION

| Group | Wt. change, gm. | Urine volume, cc. | | BUN, mgm. % at death | Deaths |
|---|---|---|---|---|---|
| | | 24 hr. | 48 hr. | | |
| *Section 1* | | | | | |
| Uninephrectomy, ligation, dehydration | − 41 (− 36– − 58) | 0.7 (0.3–1.9) | 15.9 2 animals | — | 2 on 1st day<br>1 on 2nd day<br>1 accidental |
| Uninephrectomy, ligation, dehydration, testosterone, cortisone | − 35 (− 28– − 42) | 0.4 (0.1–1.0) | — | — | 5 on 1st day<br>1 on 2nd day |
| *Section 2* | | | | | |
| Uninephrectomy, ligation, dehydration | − 18 (− 14– − 20) | 0.8 (0.3–1.6) | — | 350 (3 animals) | 3 on 1st day<br>3 on 2nd day |
| Uninephrectomy, ligation, dehydration, testosterone, cortisone | − 16 (− 12– − 18) | 0.8 (0.5–1.1) | — | 440 (1 animal) | 5 on 1st day<br>1 on 2nd day |

*Experiment 13. The Effect of Cortisone Acetate and Testosterone Propionate in Combination on the Renal Damage Caused by Limb Ligation*

The animals in this experiment were handled as before in so far as prior uninephrectomy, dehydration, and then limb ligation were concerned. The experiment was carried out in two sections, adult male rats being used in section 1, adult female rats in section 2. Testosterone was administered in oil as before (5 mgm. repeated 48 and 24 hr. prior to ligation). Cortisone acetate (2 mgm. daily beginning 24 hr. before ligation) was given as in experiment 12. Table XIII presents the data.

It is obvious that the combination of testosterone with cortisone certainly provides no amelioration of the condition. If anything, the severity of the disease was increased.

*Experiment 14. The Effect of Desoxycorticosterone Acetate (DCA) on the Renal Damage Caused by Limb Ligation*

This experiment was carried out in the same manner as the preceding steroid trials. Only one section of male rats was used since no clear-cut sex difference had been noted previously. DCA was administered as a saline suspension, 2.5 mgm. in 0.5 cc. saline being given daily subcutaneously beginning 48 hr. prior to ligation. Table XIV presents the data.

DCA was without obvious effect on the results of limb ligation in uninephrectomized dehydrated rats under the circumstances of this experiment.

*Experiment 15. The Effect of 17-OH-corticosterone Acetate (Compound F) on the Renal Damage Caused by Limb Ligation*

The efficacy of Compound F in the treatment of acute tubular necrosis in uninephrectomized, dehydrated, ligated male rats was tested in this experiment. Observations were made as usual on urine output, BUN, kidney histology, and mortality rate. In section one, six animals received Compound F (Hydrocortone, Merck) as a daily subcutaneous dose of 2 mgm. beginning 48 hr. before ligation.

Because of some promising results in the first section of this experiment, it was repeated in a duplicate section. In this section, however, 3 mgm. of Compound F were given daily beginning 72 hr. before ligation. Further, in an attempt to decrease the severity of the condition, free access to water was allowed after 48 hr. of dehydration, i.e. 24 hr. after ligation.

In this section BUN was determined only at postmortem on animals freshly dead.

Table XV presents the data.

In the first section of this experiment, Compound F appeared to have a favorable influence on the course of the renal tubular disease shown histologically as well as by the data presented above. On repetition, using a higher dose of the steroid and more favorable experimental conditions (less water deprivation) this favorable effect did not occur.

TABLE XIV

THE EFFECT OF DESOXYCORTICOSTERONE ACETATE (DCA) ON THE RENAL DAMAGE CAUSED BY LIMB LIGATION

| Group | Wt. change, gm. | Urine volume, cc. | | | BUN, mgm. % | | Deaths |
|---|---|---|---|---|---|---|---|
| | | 24 hr. | 48 hr. | 72 hr. | 24 hr. | 72 hr. | |
| Uninephrectomy, ligation, dehydration | − 21 (− 12– − 32) | 1.3 (0–2.9) | 0.6 (0–2.3) 4 animals | 25.9 * | 270 (210–360) 5 animals | 190 * | 2 on 1st day 3 on 2nd day |
| Uninephrectomy, ligation, dehydration, DCA | − 23 (− 16– − 36) | 1.4 (0.7–2.3) | 0.4 (0–2.0) 6 animals | — | 360 (300, 420) 2 animals | — | 6 on 2nd day |

* *Single determination.*

TABLE XV

THE EFFECT OF 17-OH-CORTICOSTERONE ACETATE (COMPOUND F) ON THE RENAL DAMAGE CAUSED BY LIMB LIGATION

| Group | Wt. change, gm. | Urine volume, cc. | | | BUN, mgm. % | | Deaths |
|---|---|---|---|---|---|---|---|
| | | 24 hr. | 48 hr. | 72 hr. | 24 hr. | 72 hr. | |
| *Section 1* | | | | | | | |
| Uninephrectomy, ligation, dehydration | −26 (− 24– − 28) | 0.4 (0.1–0.6) | — | — | 250 * | 130 * | 5 on 1st day<br>1 accidental |
| Uninephrectomy, ligation, dehydration, compound F | −33 (− 24– − 44) | 0.9 (0.0–1.8) | 5.1 (2.9–6.7)<br>3 animals | 14.4 (7.0–26.4)<br>3 animals | 220 (240, 190)<br>2 animals | 160 (170, 150)<br>2 animals | 3 on 1st day |
| *Section 2* | | | | | | | |
| Uninephrectomy, ligation, dehydration | −33 (− 20– − 52) | 1.3 (0.2–3.4) | 12.0 (0.0–28.0) | 21.0 (11, 31)<br>2 animals | terminal 270 (150–420)<br>3 animals | — | 3 on 1st day<br>1 on 2nd day |
| Uninephrectomy, ligation, dehydration, compound F | −31 (− 24– − 38) | 1.1 (0.4–2.6) | 9.5 (0.0–21.3) | 33.0 (18.6–47.4)<br>2 animals | 240 (170–300)<br>4 animals | — | 1 on 1st day<br>3 on 2nd day |

* *Single determination.*

## Discussion and Conclusions

The experiments reported here shed no new light on the pathogenesis of acute traumatic anuria, but they do provide some interesting data concerning certain peculiarities of this syndrome in the rat. This animal responds to a crushing injury with definite renal changes shown by an accumulation of nitrogenous wastes and histological evidence of renal damage. Surprisingly enough, this is accompanied, in its early stages, by a well defined polyuria. A high blood urea in the face of a polyuria suggests that filtered urea must be rapidly transferred back to the blood, a situation implying that some tubules have become little more than sieves or inert permeable membrances. This substantiates the suggestions of others (9) that back diffusion through damaged tubules is important in traumatic anuria.

The polyuria in the rat is quite different from the oliguria or anuria which occurs in man and in the dog and seems to represent a unique species response. (It should be remembered, however, that in man complete anuria is rare.)

Myoglobin, which some workers have considered essential in the etiology of traumatic renal failure, appears to be of little specific importance, even when given in the doses reported by others. As expected, this pigment may aggravate existing renal damage, probably by causing cast formation, but it does not appear to be of etiological significance.

The state of antecedent hydration seems to be of critical importance, for crushing injuries seem to be well tolerated in fully hydrated animals and poorly tolerated in the presence of water deprivation. Smith (9) has drawn attention to this point.

These findings suggest that anuria, while an important clinical finding, is not a universal criterion for renal damage consequent upon a crushing injury.

Increasing the area of crush by extending the limb ligation to two hind limbs was followed by an increased severity of the renal damage. If a nervous reflex only were concerned, it would perhaps be expected that once the injury was severe enough to trigger a renal response, a greater injury would not evoke a greater response. In these experiments, an increase in the area of crush on the one hand, or prior reduction in renal reserve on the other, were both followed by an increase in the severity of the disease.

Testosterone, cortisone, desoxycorticosterone, and 17-OH corticosterone were all without effect on the course of the disease. The rat does not appear to be a suitable animal for studying possible therapy, however, since the disease runs such a short course. Judging by their known actions, it may still be possible that certain steroids could shorten the repair and recovery stage. Accordingly, our studies concerning "crush syndrome" have now been transferred to the dog. Work in progress indicates that a satisfactory base for the study of potential therapy can be obtained in this species.

## Summary

Data are presented concerning the production of renal dysfunction following crushing injury in the rat.

The early stage of renal injury following crush may be characterized by polyuria in this species.

Myoglobin may aggravate the renal damage following crush in the rat, but is not essential for its production.

The state of hydration of the animal prior to and immediately following the injury appears to be of critical importance in governing the degree of renal damage which follows.

Increasing the area of crush by bilateral instead of unilateral limb ligation, markedly increases the severity of the renal damage which follows.

Preliminary reduction of renal reserve by unilateral nephrectomy markedly affects the course and severity of the disease leading to a high mortality rate.

Testosterone, cortisone, desoxycorticosterone, and 17-OH corticosterone all appear to be ineffective in ameliorating the course and severity of the disease under the circumstances of these experiments.

## Acknowledgment

The testosterone and DCA used in these experiments were supplied through the courtesy of the Schering Corporation; the cortisone and Compound F through the courtesy of Merck and Co. Ltd.

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# THE ADSORPTION OF METHYL ORANGE BY LYSOZYME[1]

By J. Ross Colvin


## Abstract

Adsorption isotherms for methyl orange on lysozyme at ionic strengths varying from 0.001 to 0.05, pH 5.5, are sigmoid. Increasing ionic strength shifts the inflection point of such isotherms to higher free anion concentrations. Binding of one methyl orange anion to a lysozyme molecule in 0.05 *M* acetate, pH 5.5, facilitates adsorption of nine others, with subsequent precipitation of the protein. This co-operative behavior is interpreted on the basis of a previously described theory of interacting hydration effects. The possible biological implications for similar systems are indicated.


## Introduction

Extensive investigations during the last decade have established that when proteins adsorb ions, the isotherm is usually a good approximation to a Langmuir hyperbola (8, 10, 11, 13, 18) and may be interpreted satisfactorily by assuming Coulomb attraction between the ion and independent charged groups on the protein. In some systems, however, electrostatic attraction is insufficient to cause adsorption, even when strengthened by van der Waal's forces and hydrogen bonding. Thus, Klotz and Walker (14) were unable to observe any adsorption of the anion of methyl orange to positively charged lysozyme at pH 7.6 although this anion is bound firmly to negative proteins under similar conditions (10). Their observations have been confirmed by later work (3) but preliminary studies at pH 5.5 (5) suggested that binding* in accordance with a sigmoid isotherm may be appreciable at lower pH's. Because sigmoid isotherms are uncommon in protein adsorption studies (7, 9) and must be a reflection of co-operative** behavior within the system (7), these preliminary observations have been confirmed and extended by work reported here. In addition, the effect of ionic strength on the adsorption isotherms at pH 5.5 has been investigated. This effect and the mode of interaction of the components of the system are interpreted in terms of a theory previously outlined (3, 5).

## Materials and Methods

The lysozyme used was a crystallized product purchased from Armour and Company, Chicago. In 0.05 *M* phosphate buffer at pH 6.8, it gave a single sharp peak in the electrophoretic apparatus and in the ultracentrifuge.

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*Contribution from the Division of Applied Biology, National Research Laboratories, Ottawa. Issued as N.R.C. No. 3174.*

* *In this report, binding and adsorption are used interchangeably. Both mean the adhesion of small molecules or ions to a protein molecule or protein particle.*

** *Throughout this paper, the word co-operative is used as in Fowler and Guggenheims "Statistical Thermodynamics". It means that the interactions of the molecules or ions under discussion are so strong that the state of any one is fundamentally influenced by which states of the others are occupied.*

A solution of the protein in distilled water had a pH 5.6 ± 0.1. Molecular weight was assumed to be 14,000 (4, 16) and concentrations were corrected for moisture as determined by drying at 100° C. *in vacuo* for 16 hr. Nitrogen content of a dried sample was 17.6%.

Methyl orange was obtained from a commercial source and purified as described previously (3, 5). The sample had an extinction coefficient of 24,000 liters mole$^{-1}$ cm.$^{-1}$ at 4600 Å in 0.05 *M* acetate buffer.

Buffers were made according to Clark (2) from reagent grade materials and diluted to the appropriate ionic strength. Ionic strengths were estimated from the molarity of sodium acetate only, neglecting the contribution of the free methyl orange and the hydrogen ion at these high pH levels. When necessary, strong electrolytes were added to bring the ionic strength to the desired level.

Since methyl orange-lysozyme complexes precipitate, as might be expected from observations on analogous systems (3, 19), adsorption isotherms were determined both by the dialysis equilibrium technique and by estimating the amount of residual methyl orange in the supernatant. Known amounts of methyl orange, protein, and buffer were placed in test tubes which were equilibrated in the thermostat, centrifuged, and reequilibrated. Concentration of free dye in the supernatant was estimated by a Beckmann spectrophotometer Model DU at 5400 Å. This precipitation technique facilited estimation of the adsorption isotherm at low ionic strengths where Donnan effects invalidate quantitative results from dialysis equilibrium methods. Preliminary trials showed that free lysozyme remaining in the supernatant did not affect the extinction coefficient to methyl orange under the conditions employed. Precipitation of the methyl orange – lysozyme complex did not affect the pH of the supernatant solution and the complex dissolved completely and rapidly upon sufficient dilution of the buffer.

In both the dialysis equilibrium experiments and those utilizing precipitation of the anion–protein complex, vials were equilibrated for six hours in a water bath maintained at 32.0 ± 0.1° C. Other details of the dialysis equilibrium experiments were as described previously (3).

The relation between amount of lysozyme precipitated and amount of methyl orange removed from solution was determined as follows. Adsorbed anion was estimated as in the precipitation technique described above. Total nitrogen determinations in duplicate on each supernatant solution, using Friedrich's method (1) to reduce the azo linkage, were corrected for nitrogen in the methyl orange remaining in solution, as estimated from optical density measurements. The residual nitrogen was expressed as protein on the basis of the nitrogen content of the sample. Lysozyme precipitated was estimated as the difference between the known initial amount of lysozyme in solution and that remaining in the supernatant. Control determinations on samples of pure methyl orange each weighing approximately 4 mgm. gave within 2% total recovery of the theoretical amount of nitrogen, using the above methods.

## Results

Adsorption (and precipitation) of methyl orange by lysozyme at pH 5.5 in acetate buffer was studied as a function of ionic strength over the range 0.001 to 0.050 by the precipitation technique. Since the pH of a solution of the sample of lysozyme in distilled water was 5.6 ± 0.1, change of pH of the solution with dilution was neglected. Results are given in Fig. 1 in which $r$, the average number of bound anions per protein molecule in the system, is plotted as a function of free anion concentration. These curves show that increasing the ionic strength of the solution has no perceptible effect at low free anion concentrations and does not change the total binding capacity of lysozyme for methyl orange under these conditions. At intermediate free anion concentrations, however, increase in the ionic strength markedly decreases adsorption of methyl orange to lysozyme. Thus the general sigmoid shape of the isotherms moves toward higher free anion concentrations as the ionic strength increases.

Adsorption isotherms for methyl orange on lysozyme at various ionic strengths, pH 5.5, as determined by dialysis equilibrium, are given in Fig. 2. That the Donnan effect increases apparent binding of anion is shown by the decreasing slope of the curves as the ionic strength increases. However, in spite of this effect, the exponential form of the isotherms at low free anion

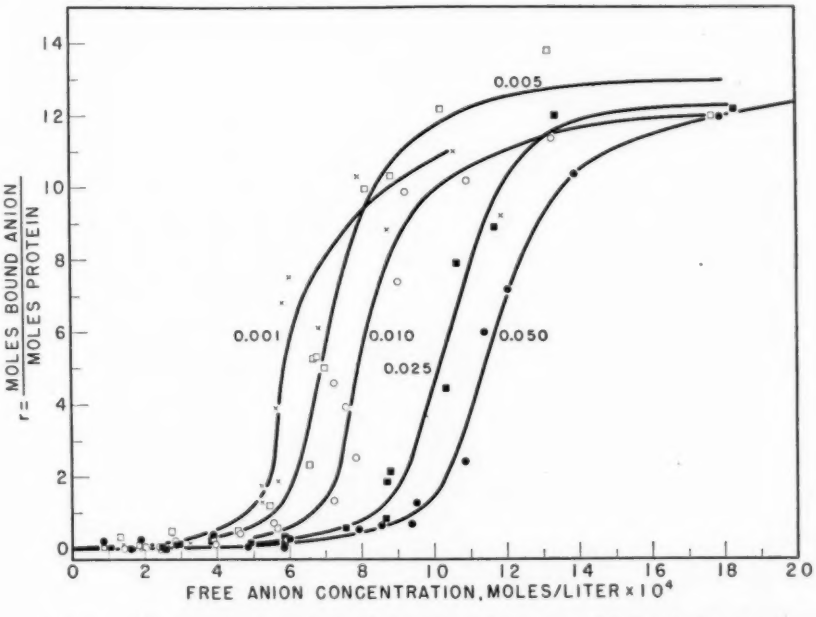


FIG. 1. Adsorption isotherms for methyl orange on lysozyme, pH 5.5, at ionic strengths varying from 0.001 to 0.050 in acetate buffer, 32° C. Isotherms estimated by the precipitation technique.

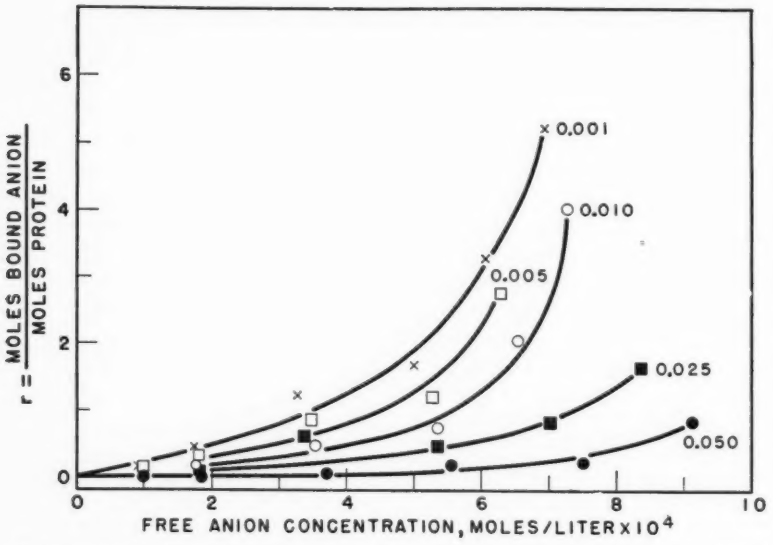


FIG. 2. Adsorption isotherms for methyl orange on lysozyme, pH 5.5, at ionic strengths varying from 0.001 to 0.050 in acetate buffer, 32° C. Isotherms estimated by dialysis equilibrium.

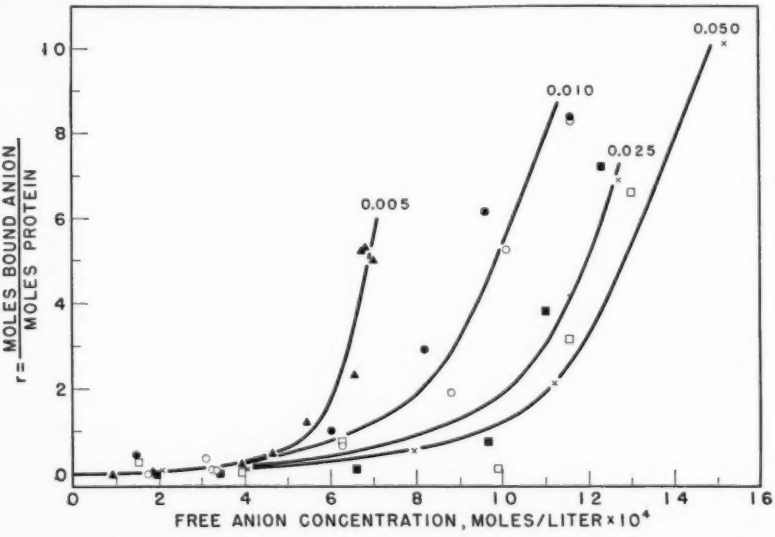


FIG. 3. Adsorption isotherms for methyl orange on lysozyme, pH 5.5, at ionic strengths varying from 0.005 to 0.050 in solutions of different anions.

▲ 0.005 acetate
● 0.009 $NaNO_3$ + 0.001 acetate
○ 0.009 $Na_2SO_4$ + 0.001 acetate
■ 0.024 $NaNO_3$ + 0.001 acetate
□ 0.024 $Na_2SO_4$ + 0.001 acetate
× 0.049 NaCl + 0.001 acetate

concentrations is unmistakable. Individual isotherms also agree well with those of Fig. 1 at the intermediate and higher ionic strengths where the Donnan effect may be neglected. These functions therefore show that the sigmoid shape of the isotherm using the precipitation technique cannot be due to lack of precipitation of soluble methyl orange-lysozyme complexes. Their concentration in the supernatant solution must be negligible. This is in agreement with observations on similar systems (3, 19).

The effect of various concentrations of sodium chloride, sodium nitrate, and sodium sulphate on the adsorption of methyl orange to lysozyme is shown in Fig. 3. Although the valence type and molarities of the salts varied, the effects of solutions of equal ionic strength were similar. In addition, although the adsorption isotherms are displaced toward higher free anion concentrations compared with those for corresponding ionic strengths in acetate, their relative position as a function of ionic strength is the same. These observations suggest that the effect of increasing salt concentation is not due to a specific ion adsorption but is a result of the variation in extent of the charge distribution about the protein molecule.

As mentioned previously, anion adsorption in this system is accompanied by precipitation of lysozyme. In such a process the amount of lysozyme precipitated by unit amount of anion adsorbed, as a function of the percentage of total binding capacity utilized, may give information about the homogeneity of the adsorption mechanism. Fig. 4 shows the amount of lysozyme

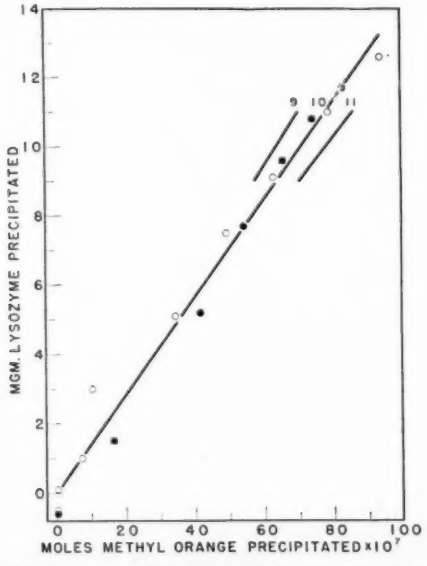


FIG. 4. Amount of lysozyme precipitated as a function of methyl orange removed from solution; 0.05 *M* acetate, pH 5.5, 32° C. Similar symbols identify points belonging to the same experiment. The two short lines show the slopes corresponding to adsorption of 9 and 11 methyl orange anions per lysozyme molecule.

precipitated as a function of methyl orange simultaneously removed from solutions which were 0.05 *M* with respect to acetate, pH 5.5, 32° C. The slope of the line is calculated from the assumption that 10 anions are removed from solution by each precipitated lysozyme molecule. The close correspondence between the line and the experimental points supports this assumption. Furthermore as shown by Fig. 5, in which the amount of lysozyme precipitated is plotted as a function of the average number of anions bound per protein molecule in the system, the above ratio is valid over at least the lower three quarters of the total binding capacity of lysozyme. Fig. 6, in which the amount of lysozyme precipitated is expressed as a function of free anion concentration, shows that within a limited range, this quantity is nearly independent of the free anion concentration. This confirms the conclusions reached from Figs. 1 and 5.

## Discussion

The isotherms of Figs. 1, 2, and 3 confirm and extend observations reported previously (5) and are of the same form as those given for similar systems (3, 19). If the attractive forces between lysozyme and methyl orange are increased by decreasing the pH and ionic strength, the anion is also bound to this protein with a sigmoid isotherm. Previous negative results (3, 14) were due to a failure to find proper conditions.

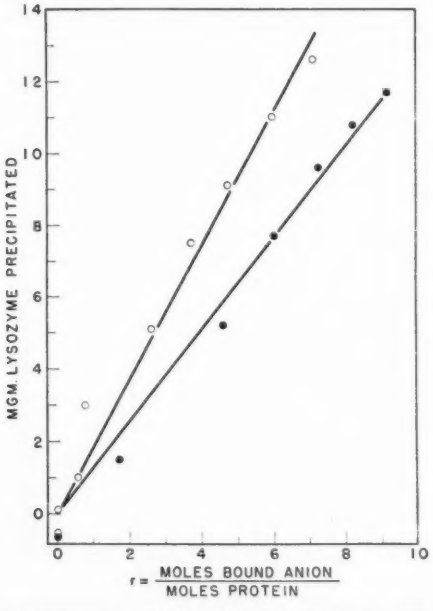


FIG. 5. Amount of lysozyme precipitated as a function of $r$, the average number of anions bound per protein molecule, in two independent experiments; 0.05 *M* acetate, pH 5.5, 32° C.

These isotherms may be interpreted by assuming interacting hydration effects on small positive proteins as described previously for similar systems (3). Such effects, if they exist, depend upon the presence of an intense, inhomogeneous, electrostatic field about each small positive protein molecule, which orients the water dipoles in the vicinity and may hinder initial adsorption of an anion, if $-\Delta F$ of hydration is large enough. As concentration of free anion increases, however, there will be an increased probability that the common Coulomb attraction, aided by variable van der Waals forces, will enable an initial anion to cross this barrier about each protein molecule. After the anion is adsorbed its presence would tend to break down the local field and facilitate adsorption of other negative ions with resultant precipitation of the lysozyme molecule–anion complex. This would be reflected in an exponential phase of the adsorption isotherm followed by a logarithmic portion when the process changes to one of random binding on a series of heterogeneous sites on the insoluble complex. As shown by Fig. 1 these expectations are consistent with the observations on the present system. Both are in agreement with the approximate general theory for such assemblies recently developed by Hill (7). Attempts to estimate the relative importance of Coulomb and van der Waals forces in such systems are now under way.

In a recent study of the interaction of methyl orange with polylysine, Wetlaufer and Stahmann (19) have reported precipitation curves of the same

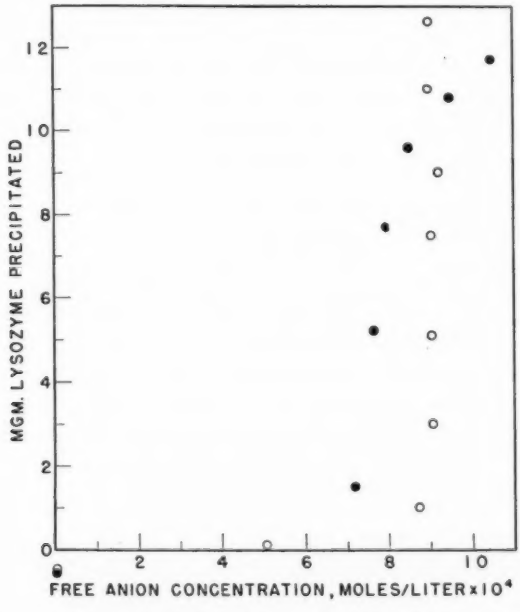


FIG. 6. Amount of lysozyme precipitated as a function of the free anion concentration in two experiments; 0.05 *M* acetate, pH 5.5, 32° C.

form as those reported here. However, they have interpreted their functions on the basis of an ideal solubility product relation, $P^{n+} + nA^- \leftrightarrows PA_n$, the initial exponential portion of the curve being attributed to polydispersity of the polylysine. In the present study, there seems little justification for assuming such marked heterogeneity of the protein, in view of the ultracentrifugal and electrophoretic data and particularly the linearity of the functions in Figs. 4 and 5. Furthermore, as mentioned above, a precipitation curve which meets the base line tangentially may still be consistent with the theory of such systems. Moreover, a formal description of the above type offers no explanation for the complete lack of intermediates $PA_x$ $(x<n)$ followed by a sharp rise in the binding curve as $PA_n$ begins to precipitate, which is characteristic of these systems. From work with similar anions on other proteins (8, 9, 11, 17, 18), intermediate complex formation would be expected to be extensive but apparently does not occur. In the light of these previous studies, the complete absence of such combinations is anomalous, if the model of Wetlaufer and Stahmann is adopted, but is wholly consistent with the mechanism of the adsorption process which was outlined above.

Although the decrease in adsorption of methyl orange by lysozyme at intermediate free anion concentrations might have been predicted by classical thermodynamics (15), it is instructive to interpret the effect for this system on a molecular basis. In the simplest case, two explanations are possible; (*a*) there is competition between the anion of the salt or buffer and methyl orange for adsorption sites or (*b*) increasing concentrations of the salt or buffer restrict the extent of the local field about a lysozyme molecule but not its total charge, leading to increased orientation of water dipoles and therefore decreased amounts of anion bound. It is assumed that the concentration of gegenions is not sufficient to completely suppress the local field of the large particle. The first explanation would imply that the much smaller acetate ion is bound to lysozyme with an intensity comparable to that of methyl orange (Fig. 1) although it has been shown that in similar systems intensity of binding usually decreases as size of the anion decreases (3). Furthermore, if the first explanation were correct, the similar effects of corresponding ionic strengths of solutions of different salts, which differ markedly in molarity and valence type, must be considered fortuitous. These considerations suggest that the second assumption of a change in extent of the charge distribution about the protein molecule, leading to a change in the $\Delta F$ for removal of a water molecule and/or adsorption of an anion, is the better one. Although this assumption agrees with the general conclusions of Wetlaufer and Stahmann from a similar study with polylysine (19), it must be regarded as tentative on the basis of present evidence.

The sigmoid isotherms of this system (Fig. 1) suggest a co-operative adsorption process in which binding of one anion facilitates the adsorption of another until the protein molecule becomes insoluble. This view is consistent with the apparent lack of intermediate complexes although lysozyme is a strongly positively charged protein with many free hydroxyls for supplementary bonding (12), as well as with the observation that each precipitated protein

molecule carries down 10 dye anions. The data of Fig. 5 provide independent confirmation of the above interpretation. If the adsorption process were random but precipitation of the protein molecule occurred whenever a critical number of adsorbed anions was exceeded (Fig. 4), a plot of protein precipitated versus $r$, the average number of anions bound per protein molecule, should be a curvilinear function, concave upwards. This conclusion follows from the fact that, in such a random process and for low values of $r$. the fraction of protein molecules carrying a given number of anions which exceeds $r$. increases approximately exponentially as a function of $r$. Fig. 5, however, is linear, within experimental error, up to $r$ values of nine* and therefore the model suggested above is inapplicable. The linearity, however, is completely consistent with the concept of co-operative binding of anions to individual protein molecules. On the other hand, Fig. 6, in conjunction with the conclusions from Fig. 2, demonstrates that little adsorption takes place until a critical concentration of free anion is reached. Clearly, adsorption of methyl orange anions to individual lysozyme molecules is dependent upon overcoming some initial barrier, after which binding is co-operative. However, the isotherms of Fig. 1 suggest that after an average of 10 anions per lysozyme molecule is exceeded, the adsorption process changes to one of random binding on a series of heterogeneous sites on the insoluble complex.

It is interesting to speculate on the possible biological implications of systems with isotherms similar to those observed here. Many instances of association between small positive proteins such as the protamines or histones and anions found *in vivo* have been described or postulated (6). If adsorption isotherms of the type described here are characteristic of similar systems a general mechanism may exist whereby effective concentrations of some constituents of cells may be maintained relatively constant with wide variations in total amounts present. Although no present evidence is known to the author to support this speculation, complexes of this sort may provide an additional buffering mechanism within cells or tissues.

## Acknowledgment

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** Whether the points in Figs. 4 and 5 are randomly distributed about a linear or curvilinear relationship is a difficult question because both measurements are subject to error and the magnitude of the errors increases as zero precipitation is approached. For Fig. 4, an approximate method of testing the fit is as follows. If the 16 pairs are separated into two equal groups, eight highest and eight lowest, and the linear regression of each group is calculated, then any curvilinearity towards the middle of the range will show up as a departure from parallelism of the two regression lines. This was done and the nonparallelism is below the 5% level of statistical significance. Therefore, there is no evidence that the relation in Fig. 4 is not linear, and, since the relations in Fig. 5 are linear transformations of that in Fig. 4, there is also no evidence that those of Fig. 5 are not linear. In any event, visual inspection of Fig. 5 will show that the apparent deviations from linearity of the two relations are opposed. Therefore a linear relation between the two variables is more acceptable than the curvilinear alternative.*

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# POLIOMYELITIS IN CANADIAN ESKIMOS

## LABORATORY STUDIES. V. TYPE 1 AND TYPE 3 POLIOMYELITIS ANTIBODY LEVELS IN BAFFIN ISLAND ESKIMOS[1]

By W. Wood,[2] Eina M. Clark,[2] F. T. Shimada[2], and A. J. Rhodes[2]


### Abstract

Studies on the basic immunology of poliomyelitis in Canadian Eskimos have been continued. Some 87 sera collected from Eskimos at Pangnirtung, Baffin Island, have been examined for the presence of Type 1 and Type 3 poliomyelitis antibody by quantitative tests in tissue cultures. The same sera were previously examined for Type 2 antibody by quantitative tests in mice. The results of the three determinations are now presented together for comparison. These sera came from Eskimos aged 2 to 72 years of age. None of the Eskimos showed any evidence of paralysis. Examination of the medical records did not suggest that any paralytic disease had been present in this part of Baffin Island. Very few of the sera showed the presence of poliomyelitis antibody; thus, Type 1 antibody was demonstrated in the sera of 8%, Type 2 antibody in the sera of 9%, and Type 3 antibody in the sera of 14%. No significant number of Eskimos below the age of 45 years had acquired poliomyelitis antibody. The antibody titers mostly ranged between $10^{-1.0}$ and $10^{-2.0}$, and were significantly lower than the titers customarily found in recently paralyzed cases. These findings suggest that poliomyelitis infection occurred in Pangnirtung Eskimos many years before the date on which the samples were taken (1951). These results point to the world-wide prevalence of the three types of poliomyelitis virus.


### Introduction

We have previously reported on the examination of the sera of healthy Eskimos living in the neighborhood of Pangnirtung, Baffin Island, for Lansing type poliomyelitis antibodies (3). This study was carried out as part of an investigation of the underlying immunology of the severe outbreak of poliomyelitis in Eskimos living on the westerly shores of Hudson's Bay in the winter of 1948–49 (1, 2, 9, 10, 11). Only 10 of 99 sera from Pangnirtung contained Lansing antibody (3). The present paper reports on the examination of 87 of these 99 sera for antibody to Types 1 and 3 poliomyelitis viruses. These antibody levels were determined in tissue cultures.

### Materials and Methods

*Preparation, Maintenance, and Infection of Cultures*

Roller-tube cultures of monkey testicular fibroblasts were prepared as described previously (11); Mixture No. 199 of Morgan, Morton, and Parker was used as the nutrient fluid (6). After five to seven days of incubation, the tissue fragments were regularly surrounded by a wide zone of fibroblasts. The nutrient fluid was then removed completely, and replaced with fresh medium; the virus inoculum was then added (11).

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*Technique of Preliminary Tests for Antibody*

All 87 sera were first tested undiluted, in order to select positive sera for later titration. A volume of 0.3 ml. of undiluted serum was mixed with an equal volume of a dilution of either Mahoney (Type 1) or Saukett (Type 3) virus in the form of a pool of infected tissue culture fluid, so that each 0.2 ml. of virus–serum mixture contained 100 $CPD_{50}$ (50% cytopathogenic doses) of virus. After the virus–serum mixtures had stood at room temperature for one and one-half hours, 0.2 ml. of each was inoculated into each of two roller-tube cultures together with 1.8 ml. of Mixture No. 199. Cultures were incubated for seven days, at which time the fibroblasts were examined microscopically for cytopathogenic changes. Sera which inhibited the cytopathogenic changes in one or both cultures were considered positive, and were frozen for titration at a later date. Appropriate virus titrations were included with each batch of these preliminary tests, in order to confirm that approximately 100 $CPD_{50}$ of virus had been incorporated in the virus–serum mixtures.

*Technique of Antibody Titrations*

Sera proving positive in the preliminary tests were diluted in 10-fold steps in Mixture No. 199, $10^{-1.0}$, $10^{-2.0}$, and $10^{-3.0}$; an equal volume of Mahoney or Saukett tissue culture virus pool was added in such a dilution that a final 100 $CPD_{50}$ of virus was present in each 0.2 ml. of virus–serum mixture. Five roller-tube cultures of monkey testicular fibroblasts were then inoculated with 0.2 ml. of each virus–serum mixture, together with 1.8 ml. of Mixture No. 199; after seven days' further incubation, the outgrowths were examined microscopically for the presence of cytopathogenic changes. Titers of serum are expressed in terms of $CPID_{50}$ (50% cytopathogenic inhibiting doses), Kärber's method being used in the calculation of the end point. A control titration of each virus pool was performed with each batch of serum tests.

Sera which, when tested undiluted, inhibited the cytopathogenic effect of the virus, but showed no inhibition at a $10^{-1.0}$ dilution, are recorded as positive (+). However, most sera inhibited the cytopathogenic effect on dilution, and the titers of these sera are expressed in the form of $CPID_{50}$ titers (in logs).

*Type 2 Antibody Determinations*

Lansing antibody determinations on these sera have been reported previously (3) but, for completeness, these results are reproduced in Table I. Briefly, the technique followed in the Lansing antibody determinations was as follows. To 10-fold dilutions of serum in saline was added an amount of a Lansing virus mouse pool sufficient to give a final 100 $LD_{50}$ in 0.03 ml. Virus–serum mixtures were left at room temperature for one and one-half hours, and were then inoculated into groups of 10 male Swiss mice intracerebrally. Mice were observed for a period of four weeks, and 50% neutralizing end points of serum were calculated by Kärber's method. The results were expressed on the following basis: A negative serum was one where the mortality in mice inoculated with the undiluted virus–serum mixture was 10/10,

TABLE I

BAFFIN ISLAND ESKIMOS: PRESENCE OF ANTIBODY IN SERUM FOR THE THREE TYPES OF POLIOMYELITIS VIRUS ARRANGED IN FIVE YEAR AGE GROUPS

| Age group (years) | Reference number | Age | Sex | 50% neutralizing end point of serum (in logs) | | |
|---|---|---|---|---|---|---|
| | | | | Type 1* | Type 3* | Type 2** |
| | E6-569 | 2 | M | – | – | – |
| | E6-547 | 3 | M | – | – | – |
| 0–4 | E6-507 | 4 | M | – | – | – |
| | E6-509 | 4 | F | – | – | – |
| | E6-522 | 4 | F | – | – | – |
| | E6-534 | 4 | M | – | – | – |
| | E6-462 | 6 | M | – | – | – |
| | E6-446 | 7 | F | – | – | – |
| 5–9 | E6-450 | 7 | M | – | – | – |
| | E6-418 | 9 | M | – | – | – |
| | E6-420 | 9 | M | – | – | – |
| | E6-136 | 10 | M | – | – | – |
| | E6-316 | 10 | F | – | – | – |
| | E6-468 | 10 | M | – | – | – |
| | E6-25 | 11 | F | – | – | – |
| | E6-354 | 11 | F | – | – | – |
| 10–14 | E6-36 | 12 | F | – | – | – |
| | E6-226 | 12 | F | – | – | – |
| | E6-281 | 12 | M | – | – | – |
| | E6-401 | 12 | F | – | – | – |
| | E6-212 | 13 | F | – | – | – |
| | E6-315 | 13 | F | – | – | – |
| | E6-297 | 14 | M | – | – | – |
| | E6-24 | 15 | M | – | – | – |
| | E6-210 | 15 | M | – | + | – |
| | E6-490 | 15 | M | – | – | – |
| | E6-191 | 16 | M | – | – | – |
| | E6-332 | 16 | M | – | – | – |
| | E6-15 | 17 | M | – | – | – |
| | E6-353 | 17 | F | – | – | – |
| | E6-30 | 18 | F | – | – | – |
| 15–19 | E6-120 | 18 | F | – | – | + |
| | E6-139 | 18 | M | – | – | – |
| | E6-164 | 18 | F | – | – | – |
| | E6-294 | 18 | M | – | – | – |
| | E6-343 | 18 | F | – | – | – |
| | E6-22 | 19 | M | – | – | – |
| | E6-190 | 19 | M | – | – | $10^{-1.0}$ |
| | E6-238 | 19 | F | – | – | – |
| | E6-400 | 19 | F | – | – | – |
| | E6-487 | 20 | M | – | – | – |
| | E6-109 | 20 | F | – | – | – |
| | E6-196 | 21 | M | – | – | – |
| | E6-326 | 21 | M | – | – | – |
| | E6-14 | 22 | M | – | – | – |
| 20–24 | E6-197 | 22 | F | – | – | – |
| | E6-280 | 22 | M | – | – | – |
| | E6-358 | 22 | F | $10^{-1.5}$ | – | – |
| | E6-393 | 22 | M | – | – | – |

* *Determined in tissue cultures.*
** *Determined in mice.*

TABLE I—(*Concluded*)

| Age group (years) | Reference number | Age | Sex | 50% neutralizing end point of serum (in logs) | | |
|---|---|---|---|---|---|---|
| | | | | Type 1* | Type 3* | Type 2** |
| 20–24 | E6-21 | 23 | F | – | – | – |
| | E6-28 | 24 | M | – | – | – |
| | E6-162 | 24 | F | – | – | – |
| | E6-222 | 24 | M | – | – | – |
| 25–29 | E6-169 | 25 | M | – | – | – |
| | E6-201 | 26 | F | – | – | – |
| | E6-392 | 26 | M | – | – | – |
| | E6-405 | 28 | M | – | – | – |
| | E6-16 | 29 | M | – | – | – |
| 30–34 | E6-314 | 31 | F | $10^{-2.7}$ | – | – |
| | E6-35 | 32 | F | – | – | – |
| | E6-69 | 32 | F | – | – | – |
| | E6-161 | 32 | F | – | – | – |
| | E6-181 | 32 | M | – | – | – |
| | E6-200 | 34 | M | – | – | – |
| 35–39 | E6-157 | 35 | M | $10^{-0.9}$ | – | – |
| | E6-68 | 37 | M | – | – | – |
| | E6-227 | 38 | M | – | – | – |
| 40–44 | E6-187 | 40 | M | – | $10^{-1.9}$ | – |
| | E6-220 | 40 | M | – | – | – |
| | E6-243 | 40 | M | – | + | $10^{-1.5}$ |
| | E6-351 | 40 | M | – | – | – |
| | E6-123 | 43 | F | – | – | – |
| | E6-221 | 44 | F | – | – | – |
| 45–49 | E6-26 | 45 | M | – | – | – |
| | E6-19 | 48 | F | – | $10^{-1.5}$ | – |
| 50–54 | E6-499 | 50 | F | $10^{-1.7}$ | – | – |
| | E6-202 | 53 | F | – | $10^{-0.7}$ | – |
| | E6-27 | 54 | F | – | – | – |
| 55–59 | E6-270 | 55 | F | – | $10^{-1.3}$ | – |
| | E6-374 | 55 | F | $10^{-1.5}$ | $10^{-1.5}$ | – |
| | E6-488 | 55 | M | – | – | – |
| | E6-489 | 56 | F | $10^{-1.3}$ | $10^{-1.5}$ | + |
| 60 and over | E6-278 | 60 | F | – | $10^{-0.7}$ | – |
| | E6-193 | 70 | M | – | – | $10^{-2.3}$ |
| | E6-208 | 71 | F | + | + | $10^{-1.2}$ |
| | E6-386 | 71 | F | – | $10^{-1.5}$ | $10^{-1.6}$ |
| | E6-207 | 72 | M | – | $10^{-0.7}$ | $10^{-1.1}$ |

* *Determined in tissue cultures.*
** *Determined in mice.*

9/10, 8/10, 7/10, 6/10, 5/10, or 4/10; a positive (+) serum was one where the mortality picture in mice inoculated with undiluted virus–serum mixture was 0/10, 1/10, or 2/10. In the case of more strongly positive sera the titers were expressed in the form of 50% neutralizing end points (in logs).

## Results

The quantitative determinations of antibody to the three types of poliomyelitis virus in the sera of the 87 Pangnirtung Eskimos are recorded in Table I. It will be noted that seven sera (8%) contained Type 1 antibody. The youngest individual with Type 1 antibody was 22 years of age; four of the seven positive sera were from Eskimos over 50 years of age. Type 2 antibody was present in the sera of eight Eskimos (9%). Two Eskimos aged 18 and 19 years had positive sera, and the remaining six Eskimos whose sera contained Type 2 antibody were all aged 40 years or over. The sera of 12 Eskimos (14%) contained antibody to Type 3 virus. The youngest individual with Type 3 antibody was 15 years old and, with this exception, the positive sera came from Eskimos over 40 years of age.

The results are therefore broadly similar for each type of antibody. It will be noted that four individuals have antibody to two types of virus, and two have antibody to all types. No correlation between the presence of antibody and family groups was apparent.

The relation between age and development of antibody is shown graphically in Fig. 1. The percentage of sera proving positive has been plotted for each 15 year age group. It will be seen that it is only after the age of about 44 that any appreciable percentage of Eskimos have poliomyelitis antibody in the serum. In the age group 45 and over, approximately 29% of Eskimos have Type 1 antibody, 35% have Type 2 antibody, and 64% have Type 3 antibody in the serum.

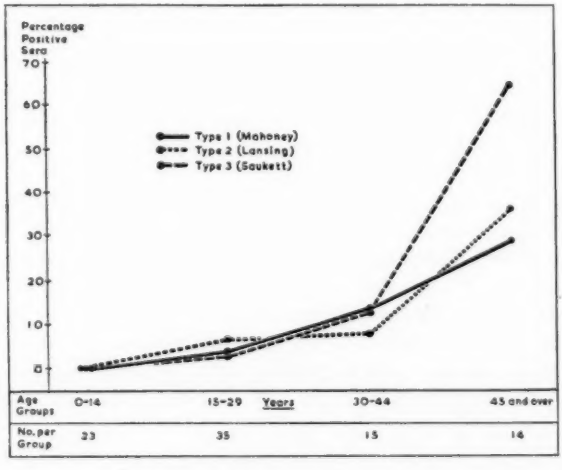


FIG. 1. Age distribution of antibody to the three antigenic types of poliomyelitis virus in sera of Pangnirtung Eskimos.

## Discussion

We have continued our study of the basic immunology of poliomyelitis in Canadian Eskimos, and in this paper we have reported on the examination of the sera of 87 healthy Eskimos from Pangnirtung, Baffin Island. These sera were tested for antibody to the three types of poliomyelitis virus in tissue culture or mice by a quantitative method.

Examination of records at the outpost hospital of Pangnirtung by Dr. J. R. Judge has failed to reveal any evidence of paralysis in Eskimos that might be attributed to poliomyelitis. No reports of paralytic illness in Eskimos in this part of Baffin Island have been obtained from other sources. However, as previously reported (3), poliomyelitis probably occurred in the settlement of Lake Harbour in the south of Baffin Island in 1942.

The Pangnirtung Eskimos represent one of the most isolated communities studied in respect to poliomyelitis antibody. Contact with the outside world is confined to one or two calls of a steamer during the brief period of open water each summer.

Our results have shown that antibody to all three types of virus was present in the sera of Eskimos, but the incidence of persons with serum antibody was low. It appeared that no appreciable percentage of Eskimos under 45 years of age had acquired antibody.

It is of interest to compare these findings with those of other workers who have examined sera for antibody to the three types of poliomyelitis virus.

Paul and his co-workers have published several studies on antibody levels in North Alaskan Eskimos. Most relevant to the present discussion is their study of the presence of antibodies to the three types of poliomyelitis virus in the sera of Eskimos collected in 1949 from 242 members of a remote population on the north coast of Alaska (8). Neutralization tests for Lansing (Type 2) antibody were carried out quantitatively in mice. Tests for antibody to Types 1 and 3 were carried out qualitatively in monkeys. Type 2 antibodies were found in high titer in individuals above the age of 20 years. Antibodies to Types 1 and 3 strains were found in an appreciable percentage of individuals over the age of 30 and 40 years respectively. The authors considered that these antibodies had persisted for 30 or 40 years in the absence of reinfection. It was suggested that exposure to the three types of virus occurred in the neighborhood of 1905 (Type 3), 1915 (Type 1), and 1930 (Type 2); no exposure had apparently taken place since those dates. The authors of this study were only able to find records of poliomyelitis in 1930 although it appears that there may have been an epidemic prior to 1921. Numerous Eskimos over the age of 20 years had antibodies to all three types. Our results would suggest that in Baffin Island, Type 1, Type 2, and Type 3 viruses were prevalent 40 or 50 years ago, and not to any appreciable degree since.

Of interest also is another study by Paul, *et al.* (7), carried out on the sera of native infants living in the vicinity of Cairo, Egypt. A serological survey of neutralizing antibodies as determined qualitatively for the three types of

virus revealed that all these antibodies were acquired at an early age among native Egyptians. More than 50% of infants had acquired Type 2 antibodies before they were two years of age, and 75% of children had acquired antibody to all three types before they were four years of age. Hammon and Sather (5) have recently reported on a study of the sera of children in two age groups (3–5 and 10–12 years) in Mexico City, Guam, and San Francisco. The results in the children in San Francisco were as follows: 11% of sera contained antibody for Type 1, 37% antibody for Type 2, and 15% for Type 3. None of the children in corresponding age groups in our series had antibody to any of the three types of virus.

Our tests for poliomyelitis antibody were carried out by quantitative methods. Of 27 titers, 10 were less than $10^{-1.0}$, 15 were between $10^{-1.0}$ and $10^{-2.0}$, and only two were above $10^{-2.0}$. Our own experience of antibody levels in recent cases of poliomyelitis would suggest that titers of $10^{-2.0}$ or greater are the rule in the early convalescent period and for at least a year afterwards. These results lead us to suggest that the majority of the Pangnirtung, Baffin Island, Eskimos acquired their infection with poliomyelitis virus many years before the time when their serum was collected (1951). This study indicated that the three types of poliomyelitis virus, all of which were originally isolated in the United States (4), enjoy very much wider distribution, having apparently penetrated to the most northerly part of Canada, within the Arctic Circle.

## Acknowledgment

We are indebted to Doctors Ella Kuitunen and J. R. Judge who collected the serum samples for this study.

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# SEROLOGICAL EFFECTS OF ETHIONINE IN THE RABBIT[1]

By Christine E. Rice, P. Boulanger, and E. Annau


## Abstract

In relation to body weight, rabbits were found to tolerate a much lower dosage of ethionine than guinea pigs. Serum complement titers fell, however, even in rabbits receiving small daily doses of ethionine, an indication of a basal disturbance in the processes of protein synthesis. Total serum protein values remained within normal limits but globulin tended to increase in relation to albumin, whereas in many of the ethionine-injected guinea pigs previously studied, serum albumin had increased in relation to globulin. In rabbits given 250 mgm. daily doses of ethionine, a definite increase in the nonprotein nitrogen content of the serum was recorded.


## Introduction

The repeated injection of ethionine has been shown to induce definite changes in the complement titer and coagulative activities of the blood of guinea pigs (13,14). It seemed of interest to determine whether this analogue of methionine would induce similar changes in another animal species such as the rabbit, whose enzymatic systems might be somewhat different from those in the guinea pig (1,5). Whereas, however, individuals of the latter species had been found to survive a daily dose of 50 or 100 mgm. ethionine for a considerable period, rabbits given a similar dosage in relation to body weight succumbed within a few days. Almost all of the rabbits that died showed gross internal hemorrhage resembling that in rabbits given large doses of dicumarol. It was not until the daily dosage had been reduced to 50 mgm. or less per rabbit that the animals withstood treatment.

## Experimental Methods

In the first experiment, four female rabbits, weighing 1860 to 2355 gm., were fed repeated doses, usually daily, of 250 mgm. ethionine, a dosage calculated on the basis of that tolerated by guinea pigs. All four animals died or were sacrificed when moribund within 13 days. Two female rabbits fed 100 mgm. ethionine daily, and four, two male and two female, fed 50 mgm. daily also died within ten days. Two rabbits fed 50 mgm., one fed 25 mgm., and one 12.5 mgm. daily remained in apparently good health for over six weeks; both were males.

The first and last series of rabbits were bled for serum complement titrations and protein analyses. Studies of plasma coagulability were also made on the last group of four rabbits. The complement titrations, two-stage prothrombin determinations, and protein analyses were made as described in our earlier studies of the effects of ethionine in guinea pigs (13,14).

## Experimental Results

All 10 rabbits that died during the course of ethionine treatment were autopsied. Almost all showed massive internal hemorrhage, usually in the

[1] *Manuscript received November 2, 1953.*
*Contribution from Animal Pathology Division, Canada Department of Agriculture, Animal Diseases Research Institute, Hull, Quebec.*

region of the kidney. Like ethionine-treated rats, mice, and guinea pigs, these rabbits had developed fatty livers, although on the whole the tendency for fat to accumulate in the liver did not appear quite so marked as that previously encountered in guinea pigs. In the latter species, a few days prior to death the serum usually contained considerable bilirubin; this was not observed in any of the rabbits in these series of experiments. The gross pathological findings for four female animals will be described briefly; the results of more detailed microscopic examinations will be described elsewhere (12).

No. 621—fed eight 250 mgm. doses of ethionine over a period of 12 days; found dead on the 13th day. At autopsy massive hemorrhage was found in the fat in the region of the kidney. The liver was fatty, the adrenals pale. The other organs appeared normal.

No. 625—fed the same dosage of ethionine as No. 621; killed after 13 days when in a moribund condition. The gross pathological findings appeared similar to those in the foregoing except for the absence of internal hemorrhage.

No. 624—fed one more dose of ethionine than Nos. 621 and 625; died in convulsions, autopsied immediately. Massive hemorrhage had occurred into the fatty tissue surrounding the right ovary; there was also some hemorrhage on the left side.

No. 631—fed 100 mgm. ethionine for eight days; died on the ninth day. On postmortem examination, massive hemorrhage was found about the right kidney and in the muscles of the thoracic cavity on both sides and at the back of this organ. The ovaries appeared normal. The liver was discolored and fatty.

*Complement Titers*

As in ethionine-treated guinea pigs, all of the similarly treated rabbits showed a fall in complement titer, particularly those on the highest dosage (Table I). All four major complement components were reduced; of these the first was least affected. Even when the dosage of ethionine was reduced to 12.5 or 25 mgm. daily, there was still a demonstrable decline in complement titer (Table II). Three of the rabbits had somewhat higher complement titers for the second than for the first bleeding. Some ability to adjust to

TABLE I

COMPLEMENT TITERS OF SERA OF ETHIONINE-TREATED AND CONTROL RABBITS

| No. of rabbit | Dose of ethionine, mgm. | No. of doses | Date of bleeding | Complement titer (units/ml.) | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | C′ | C′1 | C′2 | C′3 | C′4 |
| | | | 1952 | | | | | |
| 621 | 250 | 8 | 8/1 | 11 | 116 | <50 | 56 | <50 |
| 624 | 250 | 8 | 8/1 | 10 | 83 | <50 | <50 | <50 |
| 628 | 250 | 8 | 8/1 | 8 | 83 | <50 | <50 | <50 |
| 622 | 0 | 0 | 8/1 | 156 | 417 | 185 | 227 | 194 |
| 620 | 0 | 0 | 8/1 | 128 | 193 | 181 | 500 | 227 |

TABLE II

COMPLEMENT TITERS OF SERA AND TWO-STAGE CLOTTING TIMES OF PLASMA OF ETHIONINE-TREATED AND CONTROL RABBITS

| No. of rabbit | Dose of ethionine, mgm. | Number of doses | | Complement titer (units/ml.) | | Clotting time (sec.) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Stage I | | Stage II | |
| | | 18/2 1st Bl. | 29/2 2nd Bl. | 18/2 1st Bl. | 29/2 2nd Bl. | 18/2 1st Bl. | 29/2 2nd Bl. | 18/2 1st Bl. | 29/2 2nd Bl. |
| 666 | 12.5 | 16 | 25 | 39 | 67 | 215 | 202 | 11 | 8 |
| 667 | 25 | 16 | 25 | 28 | 63 | 96 | 204 | 8 | 9 |
| 701 | 50 | 12 | 21 | 11 | 24 | 148 | 130 | 12 | 9 |
| 702 | 50 | 12 | 21 | 31 | 11 | 156 | 1000 | 9 | 9 |
| 635* | 50 | 15 | | 19 | | >1200 | | 86 | |
| 620 | 0 | 0 | 0 | 125 | 135 | 98 | 91 | 8 | 8 |
| 626 | 0 | 0 | 0 | 122 | 139 | 106 | 119 | 9 | 9 |
| N 1 | 0 | 0 | 0 | 125 | | 146 | | 14 | |
| N 2 | 0 | 0 | 0 | 99 | | 149 | | 16 | |
| N 3 | 0 | 0 | 0 | 143 | | 112 | | 13 | |

* *Died four days later with massive internal hemorrhage.*

chronic dosage of ethionine had been previously observed in guinea pigs. One of the two rabbits surviving the 50 mgm. dosage evidently failed to show such an adjustment, its complement titer continuing to fall.

*Plasma Coagulability*

The fact that the rabbits on the higher dosage of ethionine died of massive internal hemorrhage similar to that noted in dicumarol-treated rabbits suggested that this drug might have produced a marked prolongation of clotting time such as had been recorded in the guinea pig. Plasma from bleedings from the two rabbits on the 12.5 and 25 mgm. dosage and from three on the 50 mgm. dosage were therefore tested by the modified two-stage technique used in these previous studies. In this technique, the stage-I clotting time of a 1:50 dilution of plasma is determined without added fibrinogen; in the determination of the stage-II clotting time, fibrinogen is added after an interval of 45 sec. allowed for the conversion stage. As shown in Table II, one of the rabbits, No. 635, that had been given fifteen 50 mgm. doses of ethionine, showed a marked increase in both stage-I and stage-II clotting times. The stage-I clotting times were somewhat longer than those of the controls for the second bleeding from rabbits Nos. 666 and 667, but their stage-II clotting times fell within the "normal" range. Unfortunately, tests of coagulability were not made on plasma from rabbits given the larger doses of ethionine.

*Serum Protein Analyses*

Serum protein values for eight ethionine-treated rabbits were compared with those of six control untreated animals bled on the same days (Table III). The total protein values of the sera of the eight treated rabbits were not

TABLE III

RESULTS OF PROTEIN ANALYSIS OF SERUM OF ETHIONINE-TREATED AND CONTROL RABBITS

| No. of rabbit | Dose of ethionine, mgm. | No. of doses | Date of bleeding | Non-protein nitrogen, mgm./ 100 ml. | Total protein, gm./100 ml. | Albumin, gm./100 ml. | Globulin, gm./100 ml. | A/G ratio |
|---|---|---|---|---|---|---|---|---|
| | | | 1952 | | | | | |
| 628 | 250 | 8 | 8/1 | 86.2 | 8.10 | 4.33 | 3.77 | 1.1 |
| 624* | 250 | 8 | 8/1 | 78.0 | * | 4.78 | * | |
| 631 | 100 | 8 | 18/1 | 68.0 | 7.76 | 5.24 | 2.52 | 2.1 |
| 632 | 100 | 8 | 18/1 | 48.0 | 8.51 | 5.37 | 3.14 | 1.7 |
| 635 | 50 | 8 | 28/1 | 45.5 | 7.59 | 4.78 | 2.81 | 1.7 |
| 702** | 50 | 21 | 29/2 | 30.5 | 6.74 | 5.08 | 1.66 | 3.1 |
| 667** | 25 | 25 | 29/2 | 30.5 | 7.05 | 5.28 | 1.77 | 3.0 |
| 666** | 12.5 | 25 | 29/2 | 33.0 | 7.04 | 5.26 | 1.78 | 3.0 |
| 622 | 0 | 0 | 8/1 | 33.0 | 7.20 | 5.66 | 1.54 | 3.6 |
| 619 | 0 | 0 | 8/1 | 33.0 | 7.66 | 5.46 | 2.20 | 2.5 |
| 620 | 0 | 0 | 18/1 | 28.0 | 8.01 | 6.09 | 1.92 | 3.2 |
| 626 | 0 | 0 | 18/1 | 40.5 | 8.24 | 6.60 | 1.64 | 4.0 |
| N-1 | 0 | 0 | 28/1 | 30.5 | 7.12 | 5.88 | 1.24 | 4.7 |
| N-2 | 0 | 0 | 29/2 | 25.5 | 7.42 | 5.71 | 1.71 | 3.3 |

* *Bile stained, value inaccurate.*
** *These three rabbits survived the ethionine treatment.*

significantly lower than those of the controls, even in the case of rabbits that had received the largest doses of ethionine. In the sera of the four animals given eight or nine doses of 100 or 250 mgm., and one that had received eight 50 mgm. doses, the globulin values were increased. The change in the albumin–globulin ratio was therefore in the reverse direction from that usually observed in ethionine-injected guinea pigs. A fall in albumin–globulin ratio has, however, been recorded in the serum of guinea pigs repeatedly injected with small amounts of carbon tetrachloride, an agent which produces kidney damage as well as fatty livers in this and other species.

Of considerable interest also was the relatively marked elevation in the nonprotein nitrogen content of the serum of the two rabbits fed eight 250 mgm. doses of ethionine. This change, suggestive of kidney damage, has not been detected in guinea pigs.

## Discussion

Although the general pathological picture, particularly as regards the kidney, was somewhat different in ethionine-treated rabbits from that observed in guinea pigs similarly treated, both species, when given a sufficient dosage of this methionine analogue, showed a fall in complement titer and delayed plasma coagulability. A decline in complement titer was recorded in surviving rabbits that showed no other visible signs of the drug, thus supporting our earlier suggestion that complement titration might constitute a sensitive method of detecting early disturbances in protein physiology. The efficacy of methionine or cystine and choline in preventing these blood changes in the

rabbit was not studied, although our previous investigations had shown them to have some protective properties in the guinea pig.

The observation that both guinea pigs and rabbits displayed delayed coagulability of the blood after repeated larger doses of ethionine yet only the latter species succumbed to massive hemorrhage suggested that additional factors were involved, possibly differences in the degree of endothelial injury or of hypertension developing as a result of the disturbance in methionine metabolism. Recent evidence indicating that methionine plays a very important role in wound healing and the synthesis of connective tissue (9, 11, 17) may be pertinent. Furthermore, when it is recalled that collagen has a relatively high glycine content (4), the fact that ethionine has been shown experimentally to interfere with the uptake of this amino acid (15) may assume importance. Since it has been found that the effects of ethionine on serum proteins differ somewhat in the two species of animals, its relative influence upon the production of connective tissue proteins may also differ.

The resemblance between the pathological changes observed in the ethionine-treated rabbit and those resulting from choline deficiency in the rat will be discussed by Plummer and Boulanger elsewhere (12). The possibility that the ethionine-injected rabbit might prove a useful subject for a study of the development of other conditions associated with choline deficiency such as hypertension (2,3,6,7) or atherosclerosis (8,10,16), would seem worthy of attention particularly if any evidence could be obtained of a correlation between the time of development of such changes and a decline in the complement titer or coagulability of the blood.

## Acknowledgments

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# CELL FREE "CHOLINE ACETYLASE" FROM *LACTOBACILLUS PLANTARUM*[1]

By Grace T. Girvin and J. W. Stevenson


## Abstract

The conventional methods employed to extract "choline acetylase" from animal tissues have been found not to be applicable to the extraction of the bacterial enzyme. A method has been developed for the extraction of a highly active cell free "choline acetylase" from *Lactobacillus plantarum*. The procedure involves disruption of lyophilized cells with glass powder and resolution of lipoprotein complexes through extraction of the cell debris with *n*-butyl alcohol followed by acetone drying and aequeous extraction of the enzyme from the acetone dried cell residue. The enzyme extract acetylates choline when incubated under anaerobic conditions with the substrates choline and acetate and requires the presence of ATP and cysteine. Coenzyme A markedly increases the activity of dialyzed extracts. Sodium pyruvate does not affect the synthesis of acetylcholine by the bacterial enzyme; sodium fluoride acts as a potent inhibitor. The activity of the extract is at least twice that reported for purified mammalian brain preparations and is 45% as potent as the highly active squid head ganglion enzyme.


## Introduction

The enzyme "choline acetylase" or the "choline-acetylating system" was first isolated by Nachmansohn and Machado (21) in 1943 from rat brain and from the electric organ of *Electriphorous electricus*. The enzyme has since been extracted from a variety of tissues of nervous and nonnervous origin as peripheral nerve (20), squid head ganglia (22), heart auricular tissue (3), striated muscle (17), placental and splenic tissues (7), abdominal chain of the lobster (16), abdominal ganglia of *Carcinus maenas* (27), *Tubularia*, *Planaria*, and the flat worm (24), trypanosomes (4), and recently from human erythrocytes (12).

Stephenson and Rowatt in 1947 (25) reported the acetylation of choline by the bacterium *Lactobacillus plantarum* in growing cultures and in non-proliferating suspensions. They failed, however, to obtain an active cell free preparation of choline acetylase from acetone dried preparations of the organism. Stevenson and Girvin (26) in 1950 and Girvin (10) confirmed the acetylation of choline by a strain of *L. plantarium* isolated from sauerkraut. The present report concerns the extraction of a highly active "choline-acetylating system" from this strain of *L. plantarum*.

## Materials and Methods

### *The Bacterium*

The organism was isolated from sauerkraut prepared in the laboratory. It was identified as *Lactobacillus plantarum* according to Bergey's classification (2) and shown to have the ability to acetylate choline (26). The fluid medium

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used to propagate the organism, and one in which the organism produced high yields of acetylcholine, was a modification of a medium designed by Stephenson and Rowatt (25). The medium was prepared as follows:

*A.*

| | |
|---|---|
| Tryptic digest of casein (BBL) | 1.65 gm. |
| Glucose | 1.00 gm. |
| *M* sodium acetate | 0.50 ml. |

Stock salt solution:

$KH_2PO_4$ 0.15 gm.; $MgSO_4 . 7H_2O$ 0.1 gm.; NaCl 0.15 gm.; $(NH_4)_2HPO_4$ 0.6 gm.; $FeSO_4 . 7H_2O$ 1.5 gm.; $CuSO_4 . 7H_2O$ 125 mgm. $MnSO_4 . 7H_2O$ 125 mgm.; $ZnSO_4 . 7H_2O$ 125 mgm.

| | |
|---|---|
| Made up to a volume of 100 ml. with distilled water | 5.00 ml. |
| Distilled water to | 75 ml. |

Sterilized by autoclaving at 120° C. for 20 min.

*B.*

| | |
|---|---|
| Yeast extract (Difco) | 300.00 mgm. |
| Cysteine hydrochloride | 20.00 mgm. |
| Choline chloride (Merck) | 50.00 mgm. |

Dissolved in 25 ml. of distilled water and sterilized by filtration through a sterile membranstucker (Sartoriouswerke, A., C.E. co., Gottingen, Germany). This was added aseptically to the 75 ml. of medium A to give 100 ml. of the complete medium.

Stock cultures were grown for 24–36 hr. at 28° C. and maintained for periods of one month in the cold room before subculture in a semisolid medium of the following formula:

| | |
|---|---|
| Bactotryptone (BBL) | 5 gm. |
| Yeast extract (Difco) | 1 gm. |
| Tomato juice | 10 ml. |
| Glucose | 2 gm. |
| Bacto agar (BBL) | 15 gm. |
| Distilled water to | 1000 ml. |

Dispensed in deep tubes for the preparation of stab cultures and autoclaved at 120° C. for 20 min. The stock stab cultures were used to initiate seed cultures for the inoculation of large batches of medium.

The strain of *L. plantarum* grows and acetylates choline in the fluid medium at an optimal temperature of 28° C. In culture, it produces from 4 to 8 gamma of acetylcholine (ACh) per ml. of supernatant fluid. In non-proliferating suspensions, it produces from 15 to 24 gamma of acetylcholine per mgm. dry weight of cells after three hours' incubation in a chemically defined reaction mixture containing choline hydrochloride, sodium acetate, glucose, and a citric acid buffer. Washed cells, when subjected to mechanical cell rupture or to boiling, release from 3.5 to 3.7 gamma of acetylcholine per

mgm. dry weight of cells. Over a total period of three and one-half years since its original isolation from sauerkraut, our strain of *L. plantarum* has lost none of its ability to acetylate choline.

*Assay of Acetylcholine*

The method employed to assay the production of acetylcholine was essentially that of Chang and Gaddum (6) involving the frog rectus, with certain modifications suggested by Burgen (5) which have resulted in increased sensitivity of the muscle preparation. Bioassays were carried out at a temperature of 25–30° C. The test materials were assayed in the bath, in dilutions giving responses equal to those found for standard acetylcholine within the range of 0.15 to 0.3 gamma absolute concentration in the bathing fluid. Lower values of the standard give definite but not consistently reliable responses. As a control for the possible presence of nonspecific substances which might cause muscle contraction, samples were treated at pH 8.0 for one hour after which they were readjusted to neutrality and tested in the muscle bath. This procedure destroys acetylcholine.

*Culture and Harvest of L. plantarum for the Extraction of "Choline Acetylase"*

The bacteria were grown in 20 liter lots in the fluid medium and the cultures were harvested at 24–30 hr. of growth in a Laval Continous-Flow Centrifuge. The sedimented cells were washed twice with a neutral 0.1 *M* phosphate buffer, a third time with distilled water, lyophilized and stored in evacuated,

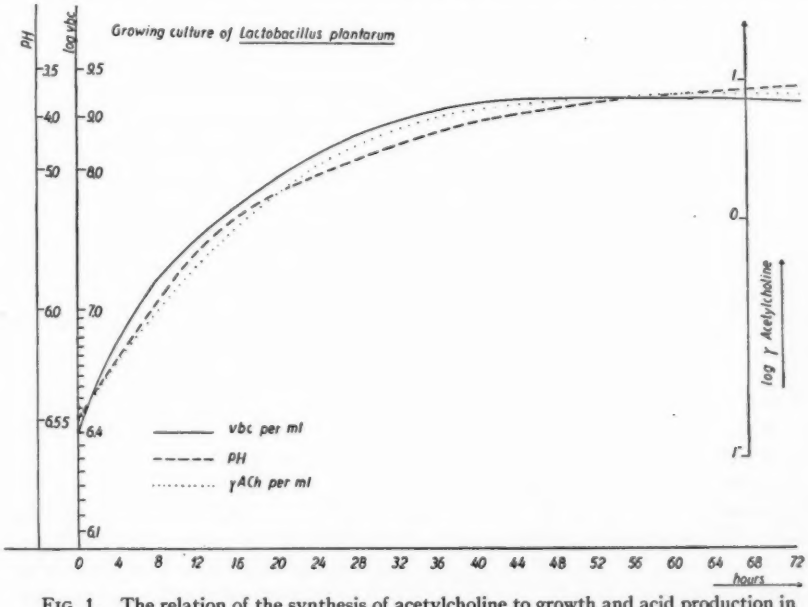


FIG. 1. The relation of the synthesis of acetylcholine to growth and acid production in *L. plantarum*.

sealed containers (vacuoliter bottles) at − 25° C. prior to extraction. A preliminary study of the production of acetylcholine in culture and its relationship to the growth curve of *L. plantarum* and pH changes in the culture over a 72 hr. period had been made. Fig. 1 illustrates that the three functions, growth, secretion of acetylcholine, and acid production are intimately related. From the data it was indicated that young 24–30 hr. cells harvested for extraction could be expected to contain an active "choline acetylase".

*The Extraction of "Choline Acetylase" from L. plantarum*

Preliminary attempts to effect an aqueous buffer extraction of the enzyme from wet or lyophilized cellular debris-abrasive mixtures, following the extraction methods employed by Feldberg and Mann (8, 9) and Nachmansohn and his co-workers (19, 20, 22) for the recovery of "choline acetylase" from mammalian brain and squid head ganglia, were consistently unsuccessful. Extracts from acetone-dried preparations of the bacterial debris also failed to exhibit activity. Simple buffer elution failed to remove any active enzyme which might be adsorbed on the cellular debris or the abrasive particles. Dialyzed extracts were inactive.

The repeated failure to obtain an active extract from the bacteria by means of the conventional techniques suggested that bacterial "choline acetylase" might be bound in a relatively undissociable complex to the cellular debris. Morton's method for the separation of enzymes from insoluble tissue particles (14) was modified to a single phase *n*-butyl alcohol extraction as the initial step in our procedure.

Lyophilized cells were ground in small aliquots with an equal weight of pulverized "resistant" glass powder in a glass mortar for 20–30 min. at room temperature. Manual pulverization of the bacterial-abrasive mixture accomplished almost total cell fragmentation as indicated by Gram stained smears. The pooled cellular debris-abrasive mixture was transferred to a round-bottomed pyrex flask with 40 gm. of 3 mm. glass beads, the tapered stopper of the flask secured in place, and the material shaken on a high speed shaking machine at 5° C. for one-half hour to remove clumps. Aliquots (20 ml.) of chilled *n*-butyl alcohol (reagent) were then added to the flask at hourly intervals over a five hour period during continued shaking in the cold room. Three grams of lyophilized ground cells were thus extracted with 100 ml. of alcohol. The mixture was permitted to remain in contact with the solvent for a period of some 16 hr. at 5° C. The solvent was separated from the mixture by filtration under negative pressure through a membrane which does not dissolve in the solvent. The extracted residue was left on the membrane and dried in a vacuum desiccator for 24 hr. A dry weight determination of an aliquot of the *n*-butyl alcohol used for extraction indicated that 2.5% of the original dry weight of the cellular debris had been assumed by the solvent. The dried residue obtained upon evaporation of the alcohol gave a negative biuret reaction whereas the extracted cell debris gave a strong biuret reaction.

To 2.8 gm. of the dried material, recovered from the membrane and gently ground to a homogeneous powder in a cooled mortar, was added 54 ml. of acetone cooled with dry ice. The acetone was added gradually over a 10 min. period with constant grinding. The mixture after being allowed to stand for 15 min. was filtered over a membrane under negative pressure. The acetone-extracted material was permitted to dry on the surface of the membrane and was then removed to a vacuum desiccator for complete drying. The powder was removed from the desiccator after a period of 24 hr., homogenized in a glass mortar, and dispensed in ampoules for lyophilization. After lyophilization, the powder was stored at − 25° C. in evacuated, sealed ampoules until aqueous extraction could be carried out. The material gave at this stage, a strong biuret reaction.

The powder was extracted at 5° C. with the following solution, allowing 1 ml. of extracting fluid for each 18 mgm. of the powder:

| | |
|---|---|
| KCl | 0.17*M* |
| $MgCl_2$ | 0.00125*M* |
| $Na_2HPO_4$ | 0.006*M* |
| (pH adjusted to 7.1, precooled to 5° C.) | |

The extracting solution was added slowly to the powder in a glass mortar with initial grinding and finally with simple stirring. The suspension was permitted to stand at 5° C. for 40 min. before centrifuging at 13,000 r.p.m. for 10 min. The supernatant fluid was decanted and filtered over a membrane. The cell-free filtrate or "extract" was dialyzed for two and one-half hours at 5° C. against distilled water, lyophilized, and stored in evacuated, sealed ampoules at − 25° C. until tested for "choline acetylase" activity.

A second extraction was carried out by the same procedure on a new harvest of cells. Nine grams of lyophilized cells were ground preparatory to the *n*-butyl alcohol extraction and a calculated weight of 48 mgm. of bacterial powder per ml. of alcohol was used. Acetone (210 ml.) was used for this batch of alcohol extracted cell debris-abrasive mixture, and aqueous extraction and recovery of a cell-free "extract" was carried out as with the first lot of material. This extract was dialyzed for 13 hr. before lyophilization.

*Preparation of a Stock, Stable Preparation of Yeast Extract as a Source of Coenzyme A (Co-E A)*

The method was essentially that employed by Kaplan and Lipmann (13) for the recovery of Co-E A from *Clostridium butyliticum*. Fleischman's "Dry Yeast Seeds" were added to 250 ml. of sterile distilled water; the mixture was autoclaved for five minutes at 120° C. following which the suspension was centrifuged at 7,000 r.p.m. for one-half hour at 5° C. The supernatant fluid was decanted and filtered with suction through a membrane. The filtrate was lyophilized and used in this form as a source of Co-E A in *in vitro* tests of

the bacterial extract for "choline acetylase" activity. Its Co-E A concentration has not been determined in a "reference" system (13) but its activity compared well with that of a commercial pig liver concentrate of Co-E A.*

*Procedure of Testing the Activity of the Cell-free, Dialyzed "Choline Acetylase" of L. plantarum*

The bacterial extract at pH 6.8 was added to the complete reaction medium presented in Table I which was adjusted to pH 7.1 before the addition of the extract. Once the extract was brought into solution from the lyophilized state it was kept cool in an ice bath prior to its inclusion in the reaction medium. The adenosinetriphosphate (ATP) was adjusted to pH 7.0 and dispensed into the small inner tube of the Myers' "Oxidation-Reduction" apparatus (15). This tube is the equivalent of the side-arm of the conventional Thunberg tube. The substrate or reaction medium was contained in the larger outer incubation tube which corresponds to the main body of a

TABLE I

"COMPLETE" REACTION MEDIUM FOR THE ACETYLATION OF CHOLINE BY A CELL-FREE ENZYME EXTRACT OF *L. plantarum*

| Constituent | Initial [*M*] | Volume in ml. | [*M*] in 2.2 ml. | Final [*M*] in 2.5 ml. |
|---|---|---|---|---|
| Extract | — | 1.0 | — | — |
| Yeast extract† or Co-E A‡ | — | 0.3 | — | — |
| Choline chloride | 0.7 | 0.1 | 0.02 | 0.0176 |
| Sodium acetate | 0.7 | 0.1 | 0.02 | 0.0176 |
| Sodium citrate | 0.5 | 0.1 | 0.015 | 0.0132 |
| Eserine | 0.052 | 0.1 | 0.0016 | 0.0014 |
| Cysteine hydrochloride | 0.7 | 0.1 | 0.02 | 0.0176 |
| Sodium phosphate | 0.0142 | 0.3 | 0.0013 | 0.0035 |
| Sodium phosphate with: | 0.007 | 1.0 | 0.0022 | |
| potassium chloride | 0.237 | — | 0.0740 | 0.0651 |
| magnesium chloride | 0.005 | — | 0.0016 | 0.0014 |
| calcium chloride | 0.004 | — | 0.0012 | 0.0011 |
| Distilled water | — | 0.1 | — | — |
| Volume | — | 3.2 | — | — |
| Volume, −1.0 ml. for preincubation bioassay | — | 2.2 | — | — |
| ATP in 0.0035 *M* sodium phosphate | 0.05 | 0.3 | — | 0.0035 |
| Final reaction volume at pH 6.9–7.0 | — | 2.5 | — | — |

**Coenzyme A concentrate "porcine origin"; 10 Lipmann units per mgm. Armour Labs. Chicago, Ill. Courtesy of Dr. W. W. Lachat.*

*† Stock yeast extract described.*

*‡ Commercial coenzyme A Concentrate (10 Lipmann units/mgm.), Armour Labs., Chicago, Ill.*

TABLE II

THE ACTIVITY OF CELL-FREE DIALYZED EXTRACTS* OF THE "CHOLINE ACETYLASE" OF *L. plantarum*

| | | (1) | | | | | (2) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Extract | | + | + | + | + | — | + | + | + | + | — |
| Yeast extract or Co-E A | | + | + | + | + | + | + | + | + | + | + |
| Choline chloride | | + | — | + | + | + | + | — | + | + | + |
| Sodium acetate | | + | + | — | + | + | + | + | — | + | + |
| ATP | | + | + | + | — | + | + | + | + | — | + |
| Gamma ACh/ml. reaction preincubation | (1) | 4.4 | 3.0 | — | 5.0 | 1.5 | 2.0 | 0.93 | 1.8 | 2.5 | 1.25 |
| | (2) | 5.0 | 3.0 | 4.8 | 5.0 | 0.8 | 2.65 | 1.83 | 2.2 | 2.5 | 0.65 |
| Gamma ACh/ml. reaction postincubation | (1) | 19.0 | 3.4 | — | 5.0 | 1.8 | 50.0 | 1.2 | 2.2 | 2.7 | 1.25 |
| | (2) | 35.0 | 3.0 | 5.1 | 5.0 | 1.1 | 50.0 | 2.0 | 2.2 | 2.7 | 1.0 |
| Gamma ACh/ml. reaction synthesized | (1) | 15.6 | 0.4? | — | — | 0.3? | 48.0 | 0.27? | 0.4? | 0.2? | — |
| | (2) | 30.0 | — | 0.3? | — | 0.3? | 47.3 | 0.17? | — | 0.2? | 0.35? |
| Gamma ACh/ml. extract synthesized | (1) | 20.7 | — | — | — | — | 67.2 | — | — | — | — |
| | (2) | 42.6 | — | — | — | — | 68.2 | — | — | — | — |

(1) *"Choline acetylase" from first extraction.*
(2) *"Choline acetylase" from second extraction.*
* *Extracts incubated anaerobically in "Complete" reaction medium at 28° C. for one hour.*

Thunberg tube. A 1.0 ml. portion of the extract-medium solution was removed from each tube for treatment and storage until a preincubation bioassay could be carried out. The tubes were then attached to the ramp of the apparatus, sealed, and evacuation and replacement of the air with nitrogen was carried out as prescribed by Myers (15). The ATP was then admixed with the contents of the main reaction tubes and incubation was carried out in the water bath at 28° C. for one hour. Following incubation, the contents of each tube were treated to stabilize acetylcholine and stored until bioassays could be carried out.

## Results

Assays of the choline acetylating activity of two cell-free extracts of *L. plantarum* are presented in Table II. It is apparent that both extracts contain a "choline-acetylating system" which requires the presence of the substrates, choline and acetate, and the presence of ATP as a source of energy for the acetylation of choline. The addition of coenzyme A to the reaction medium increases the activity of the extracts as is indicated in Table V.

The greatly increased bioassay values (Table II) for the postincubation samples containing all the elements of the complete system are interpreted as positive evidence that an *in vitro* synthesis of ACh occurred during the incubation period. The small and inconsistent increased bioassay values for the postincubation samples which lack one element of the complete system (controls) are more difficult to evaluate. It is seen that such an increase is found in samples in which the extract was not present. The interpretation of the slight and erratic increases in the concentration of acetylcholine in the other control samples must be made with reference to this finding for the control without the extract. It is considered that the increases in the post-incubation controls are more likely to be explained as apparent rather than as actual evidence of *in vitro* synthesis of ACh during incubation. It is considered most important to recall that the ACh content of samples is determined here by assay in a functional biological system. The possible contribution, made by the frog rectus muscle itself, to the ACh values obtained for the low dilutions of the control samples should be recognized.

Table III is offered for comparing the activity of the two extracts from *L. plantarum* with the activities published for "choline acetylase" preparations from mammalian brain and squid head ganglion tissue. The activity of the "choline acetylase" of *L. plantarum* has been expressed in four terms. The first two values in the table, mgm. ACh formed per gm. of "powder" extracted per hour, and the mgm. ACh formed per gm. of protein of the extract per hour, are those published by Nachmansohn and Wilson in 1951 (23) for the mammalian brain and squid head ganglia enzyme. The extracts of *L. plantarum* were lyophilized and therefore the activity for a weighed amount of the extract is given as the third term. Augustinsson has suggested in his review *The enzymes* (1) that an activity value per ml. of "choline acetylase" extracts would be desirable and thus the fourth term expresses this value for the extracts of *L. plantarum*.

TABLE III

THE COMPARATIVE ACTIVITY OF "CHOLINE ACETYLASE" FROM VARIOUS SOURCES

| Preparation* | ACh, mgm./gm./hr. | ACh, mgm./gm. dried "extract"/hr. | ACh, mgm./gm. protein/hr. | ACh, gamma per ml. "extract" |
|---|---|---|---|---|
| Fresh brain extract | 0.15–0.2 | — | — | — |
| Acetone-dried brain powder | 0.6 –1.0 | — | — | — |
| Acetone-dried brain powder, more active | 2.0 –3.0 | — | 6.0– 7.0 | — |
| Acetone-dried brain powder, purified | — | — | 25.0– 30.0 | — |
| Squid ganglion actone-dried powder | — | — | 140.0–150.0 | — |
| *L. plantarum* n-butyl alcohol extracted, acetone-dried | | | | |
| (1) | 3.0 –6.0 | 11.4–23.4 | 21.8– 44.8** | 20.7–42.6 |
| (2) | 6.6 | 31.5–32.0 | 62.2– 63.2** | 67.2–68.2 |

* *All preparations except that from L. plantarum reported by Nachmansohn and Wilson (1951).*

** *Based on micro-Kjeldahl determination.*

The "choline acetylase" of *L. plantarum* when expressed in mgm. ACh formed per gm. of powder extracted is at least as active as the complete system from the acetone-dried brain powder, probably twice as active. The value for the bacterial powder is a calculated one, and considerable manipulation of the lyophilized cells to the stage where the aqueous extraction is carried out reduces this value to limited usefulness for comparative purposes.

TABLE IV

PERCENTAGE PROTEIN CONSTITUTION OF "CHOLINE-ACETYLATING" EXTRACTS OF *L. plantarum*

| | Extract 1 (after 2½ hr. dialysis) | Extract 2 (after 13 hr. dialysis) |
|---|---|---|
| Mgm. dry weight per ml. | 3.2 | 5.1 |
| Mgm. TCA precipitated "protein" per ml. | 1.7–2.0 | 2.4–2.8 |
| Micro-Kjedahl determination of mgm. protein/ml. | 1.7225 | 2.5395 |
| Mgm. total nitrogen/ml. | 0.8321 | 0.8038 |
| % Dry weight of "dried" extract as protein* | 53.7 | 50.0 |

* *Estimated from value obtained in micro-Kjeldahl determination of protein.*

The activity of the bacterial extract is more readily appreciated from a consideration of the comparative values in terms of mgm. ACh formed per gm. of protein per hour. The first bacterial extract is at least as active, if not more active, than the purified brain extract; the second bacterial extract is twice as active as the purified brain extract and about 45% as active as the highly active squid ganglion preparation. The values for the bacterial extract in the third and fourth terms are not inflated values. The dried extract was dissolved at the time of testing to the original volume of the extract obtained before dialysis.

The protein content of the extracts (Table IV) was determined by two methods; (*a*) by a dry weight determination of the precipitate recovered after acidification of an aliquot of each extract with trichloroacetic acid; (*b*) by a micro-Kjedahl determination of the nitrogen concentration of the precipitate from a trichloroacetic acid treated aliquot of each extract.

A preliminary characterization of the "choline-acetylating system" of *L. plantarum* was initiated. The results are presented in Tables V, VI, and VII. Table V presents the results obtained with and without a source of Co-E A included in the reaction medium. There is an indication that after two hours of dialysis, some activity of extract 1 has been dissipated, which is due probably to the loss of dialyzable Co-E A. The extract shows an enhanced activity of 23 to 37% upon addition of the yeast extract. The activity of the second extract is increased to 51% by both the yeast preparation and by the pig liver Co-E A concentrate. It is rather unique that the bacterial extract 2 was not inactivated completely by 13 hr. of dialysis. It should be noted that citrate was present in the controls without Co-E A. Its possible 'activating' effect upon the extracts has not been investigated.

As can be seen from Table VI, the presence of pyruvate did not inhibit the activity of the bacterial extract but the presence of cysteine was essential to activity despite the fact the reaction was carried out anaerobically. The extract is subjected to considerable oxidation during the course of manipulation and particularly during dialysis and it is possible that sulphydryl groups on the enzyme might suffer alteration. It is also possible that the effect of the cysteine is directed towards such sensitive areas on the coenzyme itself, in view of the recent concept of the nature of the coenzyme A (Nachmansohn and Wilson, 1951) (23).

Table VII presents the results obtained when sodium fluoride was added to the mixture in contrast to a similar mixture without fluoride. The highest activity in the samples was found for the control without fluoride. The inhibitory effect of fluoride upon the extract was examined. The compound in the concentration used in the reaction had no effect upon the frog rectus response to standard ACh dilutions used for assay. The effect of sodium fluoride is thus specific in the reaction *in vitro*. It is not known whether the inhibition can be directly attributed to the fluoride ion or whether a non-specific effect of excess sodium ions is the responsible factor.

TABLE V

THE IMPLICATION OF Co-E A IN THE *in vitro* SYNTHESIS OF ACETYLCHOLINE BY BACTERIAL "CHOLINE ACETYLASE"*

| | Concentration per ml. reaction medium | Extract 1 (1.3 mgm. "dried" extract per ml. reaction medium) | | | | Extract 2 (1.5 mgm. "dried" extract per ml. reaction medium) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | (1) | | (2) | | (1) | | (2) | | (3) | |
| Extract | | + | + | + | + | + | + | + | + | + | + |
| Yeast extract or Co-E A conc. | *4.35* mgm. | + | — | + | — | + | — | | | | |
| | 5 Lipmann units (0.5 mgm.) | | | | | | | + | — | + | — |
| Gamma ACh/ml. preincubation | | 5.0 | 5.0 | 4.4 | 5.7 | 2.0 | 2.5 | 2.65 | 2.25 | 2.0 | 2.45 |
| Gamma ACh/ml. postincubation | | 35.0 | 28.0 | 19.0 | 15.0 | 50.0 | 26.0 | 50.0 | 26.5 | 51.0 | 26.0 |
| Gamma ACh/ml. synthesized | | 30.0 | 23.0 | 14.6 | 9.3 | 48.0 | 23.5 | 47.35 | 24.25 | 48.0 | 23.55 |
| Gamma ACh/mgm. extract synthesized | | 23.1 | 17.7 | 11.2 | 7.1 | 32.0 | 15.6 | 31.35 | 16.15 | 32.0 | 15.7 |
| % Activation of added Co-E A | | 23.4 | | 37 | | 51 | | 49 | | 51 | |

* *Incubated anaerobically in reaction medium for one hour at 28° C.*

TABLE VI

THE EFFECT OF SODIUM PYRUVATE AND CYSTEINE UPON THE *in vitro* SYNTHESIS OF ACETYLCHOLINE BY BACTERIAL "CHOLINE ACETYLASE"*

| | Concentration per ml. reaction medium | Extract 1 (1.3 mgm. "dried" extract per ml. reaction medium) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | (1) | | | (2) | | | (3) | | |
| Extract | 1.3 mgm. | + | + | — | + | + | — | + | + | — |
| Cysteine | 0.0176*M* | + | + | + | + | + | + | + | — | + |
| Sodium pyruvate | 0.005*M* | — | + | — | — | + | — | | | |
| Gamma ACh/ml. preincubation | | 4.4 | 5.7 | 1.5 | 5 | 5 | 0.8 | 5 | 5 | 0.8 |
| Gamma ACh/ml. postincubation | | 19.0 | 20.0 | 1.5 | 35 | 35 | 0.8 | 35 | 5 | 0.8 |
| Gamma ACh/ml. synthesized | | 14.6 | 14.3 | — | 30 | 30 | — | 30 | — | — |
| Gamma ACh/mgm. extract synthesized | | 11.2 | 11.0 | — | 23.1 | 23.1 | — | 23.1 | — | — |
| % Inhibition without cysteine | | | — | | | — | | | 100 | |
| % Inhibition with pyruvate | | | — | | | — | | | — | |

* *Incubated anaerobically in reaction medium for one hour at 28° C.*

TABLE VII

THE EFFECT OF CYSTEINE AND SODIUM FLUORIDE UPON THE *in vitro* SYNTHESIS OF ACETYLCHOLINE BY BACTERIAL "CHOLINE ACETYLASE"*

| | Concentration per ml. reaction medium | Extract 2 (1.5 mgm. "dried" extract per ml. reaction medium) | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | (1) | | | | (2) | | | |
| Extract | 1.5 mgm. | + | + | + | — | + | + | + | — |
| Cysteine | 0.0176*M* | + | — | + | + | + | — | + | + |
| Sodium fluoride | 0.0194*M* | — | — | + | — | — | — | + | — |
| Gamma ACh/ml. preincubation | | 2.0 | 2.2 | 1.33 | 1.25 | 2.65 | 2.3 | 2.2 | 0.67 |
| Gamma ACh/ml. postincubation | | 50.0 | 2.1 | 7.75 | 1.25 | 50.0 | 2.2 | 4.2 | 0.67 |
| Gamma ACh/ml. synthesized | | 48.0 | — | 6.42 | — | 47.35 | — | 2.0 | — |
| Gamma ACh/mgm. extract synthesized | | 32.0 | — | 4.3 | — | 31.5 | — | 1.3?— | — |
| % Inhibition without cysteine | | 100 | | | | 100 | | | |
| % Inhibition with sodium fluoride | | 86.7 | | | | 96 | | | |

* *Incubated anaerobically in reaction medium for one hour at 28° C.*

## Discussion

As has been mentioned earlier all our efforts to effect an extraction of the bacterial "choline acetylase" from acetone-dried preparations of *L. plantarum* were unsuccessful. It is felt that the successful aqueous extraction of the enzyme depends on the primary extraction of lipid material from the cellular debris with *n*-butyl alcohol. The secondary drying or extraction with acetone may have had the additive effect of removing lipid but it may also have served, as was intended, to help remove residual alcohol from the powder and to stabilize the powder by mediating subsequent hydration during manipulation. A determined weight was assumed by the *n*-butyl alcohol used for extraction (2.5–3.0% of the original weight of the lyophilized cell preparation). The dried residue of the alcohol gave a negative biuret reaction. An identification of the residue has not as yet been attempted. It would be most interesting to study the nature of such extracted bacterial material and since the technique is quite simple, the method might be adapted to carry out an investigation of the comparative yields from various bacterial species.

It is proposed that the extraction of the bacterial "choline acetylase" depends primarily upon its presence in sufficient concentration within the intact cells, and secondarily upon its successful resolution from a lipoprotein complex binding the active protein material to the cellular debris. It is not known whether this complex exists as a part of the cell wall or plasma membrane or whether it is a true intracellular entity.

The bacterial enzyme is typical of the "choline acetylase" recovered from other sources in its essential requirements for *in vitro* activity. The substrates choline and acetate are required as well as the presence of adenosinetriphosphate and coenzyme A during anaerobic incubation of the bacterial extracts in the reaction medium. Cysteine is required for the dialyzed bacterial extracts, probably, as in the mammalian extracts, for the reduction of oxidized sulphydryl groups in the system. The bacterial extracts are not purified preparations and may contain a transacetylating system as well as a specific "choline acetylase". The system functions at a pH of 6.8–7.0 at 28° C. which is the temperature at which actively growing cells of *L. plantarum* acetylate choline in culture.

It is unique that the extracts are not completely inactivated by dialysis for 13 hr. Presumably, some coenzyme A is lost during dialysis since both yeast extract concentrate and the pig liver coenzyme A do heighten the activity of the extracts considerably. The dialysis of the extracts would seem to be efficient, 93 and 97% of their "preformed" acetylcholine content being removed during the process. If the bacterial coenzyme A exists as an unbound compound and is dialyzable, it is difficult to understand why a critical amount was not lost after prolonged dialysis.

It is not known whether the ions of magnesium, potassium, and calcium are required for activity in the bacterial extracts. During the course of this investigation, these ions have always been included in the reaction medium.

The absence of any effect of sodium pyruvate upon the *in vitro* synthesis of acetylcholine cannot be evaluated until further investigation is carried out. The recent work of Harpur and Quastel (11) on carbohydrate interference with acetylcholine synthesis in cell-free extracts of brain indicates that a comparable examination of the bacterial extracts might be well advised. The almost complete inhibition of the bacterial enzyme by sodium fluoride was unexpected since it has not been reported for mammalian preparations. Further investigation of this effect must be carried out before it can be concluded that such inhibition is caused by the fluoride ion rather than by a nonspecific effect of excess sodium ions.

The bacterial extracts have retained full activity for over four months when stored in the lyophilized state in evacuated ampoules at − 25° C. The relatively high activity of the two unpurified extracts has already been indicated. The actual "choline acetylase" content must be only a fraction of the 50 to 53% weight of protein in the lyophilized extracts. A high activity for the bacterial extracts is perhaps expected, since the cells of *L. plantarum* produce 1.5–2.4% of their dry weight in secreted acetylcholine when incubated with glucose in nonproliferating suspensions (26, 10). If the bacterial acetylation of choline is linked primarily or secondarily with glucose utilization during growth as is suggested in Fig. 1, a highly active "choline-acetylating system" could be expected in the efficient lactic acid producer, *L. plantarum*. Stephenson and Rowatt (25) who demonstrated the dependence of *L. plantarum* upon glucose for acetylcholine secretion in nonproliferating suspensions, also determinated that 73 to 80% of the glucose provided for their strain of *L. plantarum* in culture was converted to lactic acid.

In conclusion, it is felt that the preliminary steps taken to characterize the "choline-acetylating system" of *L. plantarum* might be profitably extended following purification of the bacterial enzyme by the procedure used by Nachmansohn, Hestrin, and Voripaieff (18) for mammalian nervous tissue.

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